LXVI. ON THE PHOTO-OXIDATION OF ADRENALINE.

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In preliminary experiments it was observed that the presence of a very small amount of adrenaline caused blood-plasma to absorb in ultra-violet light about four times as much oxygen as the plasma alone. It was therefore decided to test the behaviour of adrenaline in ultra-violet light, employing the technique devised by Harris [1926]. Two quartz tubes, one for the adrenaline and the other the control, coupled to a Haldane volumeter were irradiated with a mercury-vapour lamp ($\frac{1}{2}$ kw.) at a distance of 15 cm. The tubes were kept in a bath at 37°. Different preparations of adrenaline were used: adrenaline hydrochloride in solution (Parke, Davis) and pure adrenaline (Parke, Davis), dissolved either in distilled water or in 0·5 % hydrochloric acid. Amounts of 0·0005 to 0·004 g. adrenaline dissolved in 45 cc. distilled water were used.

In the first place it was determined whether oxygen absorption takes place in the dark. The volume in the adrenaline tube remained unchanged when 0·0005 to 0·004 g. adrenaline was used. Pure adrenaline, dissolved in distilled water, only showed occasionally a minute uptake of oxygen. The solution became more or less pink after a long exposure to ultra-violet radiation. The intensity of the pink colour is in direct relation to the amount of oxygen absorbed by the adrenaline.

The oxygen uptake by different amounts of adrenaline was next determined. The adrenaline hydrochloride solution in volumes from 0·5 cc. to 4 cc. was used; this solution is called A. The results are shown in Fig. 1. The higher the concentration of the adrenaline the greater the absorption of the oxygen. But the amount of the oxygen is not in direct proportion to the amount of adrenaline. The oxygen uptake by adrenaline of a higher concentration is relatively less. It is, however, probable that the absorption would be greater after a longer irradiation, and if the partial pressure of oxygen in the tubes were maintained at the same level throughout the experiments.

In the next experiments the different intensities of oxidation of different kinds of adrenaline under various conditions were tested. Here 0·001 g. of adrenaline was used and irradiated for 30 minutes. Pure adrenaline, dissolved in distilled water (C), has a very intense oxidation (Fig. 2).

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It was further shown that hydrochloric acid is a good protective against photo-oxidation even in very low concentration (0·5 %). After an irradiation of 30 minutes adrenaline (C) took up six times more oxygen than adrenaline dissolved in 0·5 % HCl (B). And even after switching off the light the oxidation of the adrenaline C continued, though not so intensely (Fig. 2).

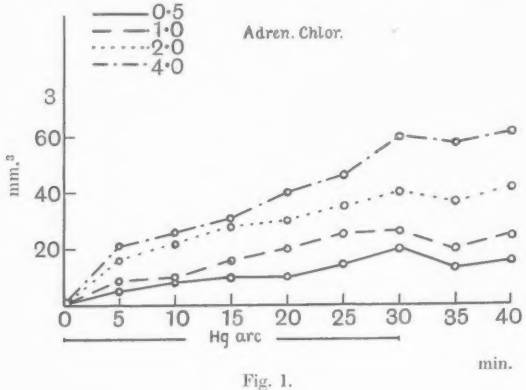

Fig. 1.

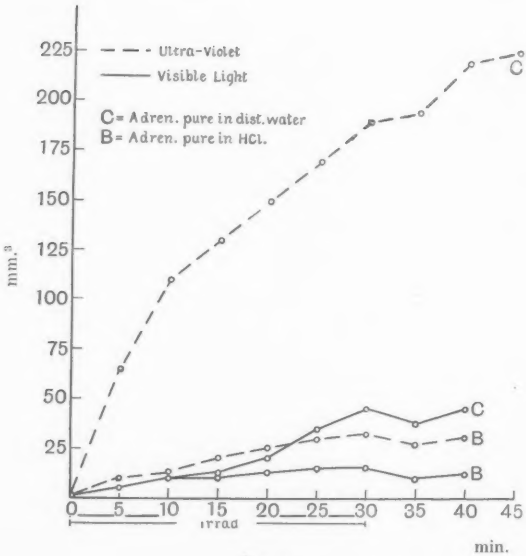

Fig. 2.

It was necessary to examine the behaviour of hydrochloric acid itself in the ultra-violet radiation. It was found that neither distilled water nor the

amount of HCl here used (1 cc. 0·5 % HCl) absorbed any appreciable amount of oxygen after a 30 min. exposure to the radiation of a mercury-vapour lamp.

It is well known that adrenaline oxidises in daylight and in these investigations it was also the object to determine the degree of oxidation in an intense light but without ultra-violet rays. In order to filter the short-wave rays a triple plate-glass filter was put between the source of the radiant energy and the quartz tubes. The two solutions (B, C) of adrenaline (0·002 g.) were examined and the results are given in Fig. 2. An oxidation took place even in the visible light, though it was much less than that caused by ultra-violet rays. The greatest uptake of oxygen in the presence of visible light only was observed in the adrenaline solution (C), whilst (B) seems to be very resistant to the influence of visible light.

Conclusions.

Different preparations of adrenaline show different tendencies to oxidation. Ultra-violet radiation causes an acceleration of the oxidation of adrenaline even in very large dilutions. The greatest sensitivity to light is shown by pure adrenaline in distilled water. Adrenaline hydrochloride solution shows a very low degree of oxidation.

Adrenaline exposed to visible light only behaves similarly, but the oxidation is less intense than when exposed to the short-wave rays.

The pink colour developed by adrenaline when exposed to the ultra-violet radiations is related to the uptake of oxygen. The more oxygen absorbed by the adrenaline the more pink it becomes. It would be interesting to examine the physiological action of this adrenaline, changed by the absorption of oxygen. In the paper of Azuma and Hill [1926] there appeared a short note that adrenaline, after exposure to the light, becomes inactive.

In experiments in which adrenaline from tablets (Parke, Davis, and Burroughs, Wellcome) was used, the oxygen uptake was very intense, but it is uncertain whether this may not be caused by unknown admixtures.

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LXVII. STUDIES ON THE GROWTH OF YEAST.

IV. A NEPHELOMETRIC METHOD OF COUNTING YEAST SUSPENSIONS.

By GEOFFREY LEWIS PESKETT.

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Report to the Medical Research Council.

(*Received March 17th, 1927.*)

Below I have recorded details of a convenient method for determining the concentration of cells in suspensions of yeast. It is based on nephelometric comparison of the yeast with standard suspensions of barium sulphate. McFarland [1907] has described a rough method for the estimation of the number of cells present in bacterial suspensions, which is essentially similar. In his earliest experiments he compared the suspensions against a dark background, but subsequently he designed an apparatus in which they were observed by direct illumination. Richards and Wells [1905], in improving the earlier method of Stas [1894], found that greater accuracy could be obtained with indirect illumination and varied the amount of light which was allowed to fall on to the tubes containing the suspensions. They determined the relative lengths of tube which were exposed to illumination when the turbidities appeared equal. In the method which I am about to describe, the tubes receive equal illumination and are compared against a dark background. A series of standard suspensions is used.

The method possesses the following advantages.

(1) It is rapid and reasonably accurate.

(2) It can be applied to the direct estimation of the number of cells present in growing cultures of yeast, and observations can be made on the cultures while growth is in progress without risk of exposure to infection.

(3) No expensive or elaborate apparatus is required.

(4) Although the preparation of a series of standard suspensions takes a considerable time, these standards appear to keep indefinitely. I have in use at the moment a series which was prepared eighteen months ago.

Standard suspensions of barium sulphate.

General considerations. The conditions employed in the precipitation of barium sulphate have been shown by Folin [1905] to influence to an important extent the character and composition of the precipitate obtained. In the

preparation of the standard suspensions every effort was made to keep the conditions absolutely uniform, in order to secure uniformity in the precipitates. Moreover, the special conditions recommended by Folin for the precipitation of potassium sulphate solutions have been adhered to as closely as possible.

To avoid the errors which would occur in the measurement and dilution of suspended barium sulphate, each standard suspension was prepared separately.

The test-tubes selected to contain the standards, and those used for the growth of yeast cultures, were of uniform bore.

While the excess of barium in the suspensions was probably adequate to prevent the growth of any organism, mercuric chloride was added as an additional precaution. Denis [1921] has shown that this does not interfere with the precipitation of barium sulphate. The mercuric chloride which I employed gave no precipitate on addition of acidified barium chloride solution.

For the sake of convenience I have considered as a dissolved substance the barium sulphate present in a suspension, and expressed its "concentration" in terms of molarity. If m be used to express molar concentration $\times 10^{-5}$, or $M/100{,}000$, then the concentrations of barium sulphate in the series of standards which I have used are as follows:

1000 m, 900 m, 800 m, 700 m, 600 m, 550 m, 500 m, 450 m, 400 m, 350 m, 300 m, 250 m, 200 m, 160 m, 100 m, 80 m, 60 m, 40 m, 20 m, 10 m.

The cloudiness of any suspension containing less than 10 m $BaSO_4$ is too slight for accurate comparison with yeast suspensions. Concentrations greater than 1000 m have not been employed, as the precipitate settles too rapidly. This difficulty might have been overcome by the addition of ammonium nitrate, as suggested by Denis.

Method of preparation. The solutions employed were:

barium chloride, approximately $M/4$,
mercuric chloride, saturated solution,
potassium sulphate $M/40$ and $M/200$ (Kahlbaum).

From the two solutions of potassium sulphate further dilutions were made, namely, $M/2000$ from $M/200$, and $M/400$ and $M/4000$ from $M/40$. Each suspension was prepared as follows. To 5 cc. mercuric chloride solution in a 250 cc. flask I added successively 2 cc. pure concentrated hydrochloric acid, the requisite volume of potassium sulphate solution, and sufficient barium chloride solution to give an amount of barium in solution at least equal to that present in the precipitated form. For example, in preparing 200 m $BaSO_4$ I used 100 cc. $M/200$ potassium sulphate and added 5 cc. barium chloride. The barium chloride was added drop by drop at the rate of 2·5 cc. per minute, without shaking or moving the mixture in the flask, which was allowed to stand undisturbed for a further five minutes. The flask was then filled to the mark with distilled water, shaken very thoroughly, and a sample was transferred as rapidly as possible to a clean test-tube. The tube was immediately sealed in a flame.

Apparatus for comparing turbidities of suspensions.

For comparing turbidities I have used the apparatus shown in Fig. 1. When in use, the hinged lid (*F*) is closed and observation is made through the apertures in it (*A*). As a rack to hold the tubes I have used a modified Cole and Onslow's comparator (*B*) [Cole, 1926] in which the four compartments employed are as close together as possible, and are drilled out through the bottom to allow light to pass up from below. All the inner surfaces of the comparator as well as the apertures at the back of the posterior compartments are covered with matt-surfaced black paper. As a source of light (*C*) I have used a 40-watt gas-filled lamp with opal globe, but owing to the heat it produces I have found it necessary to cool the whole apparatus by means of an air current from an electric fan. To secure approximately equal illumination of the tubes I have inserted between the comparator and the lamp four pieces

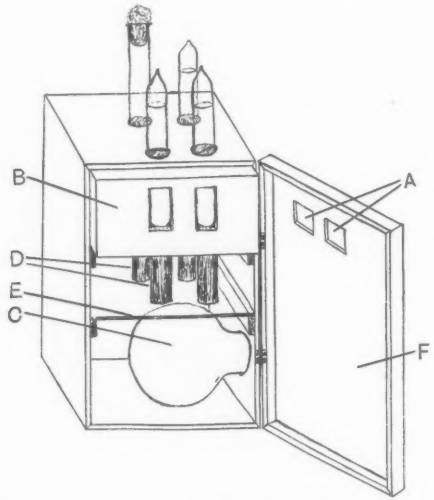

Fig. 1.

of glass tubing (*D*) of uniform length and diameter. These are lined with black paper and rest below on a glass plate (*E*). Above, they are fixed into the compartments of the comparator by means of rubber tubing. The lamp is arranged so that two samples of a suspension of yeast (or barium sulphate) display the same turbidity when compared in the apparatus; this being considered as a sufficiently accurate criterion of equal illumination of the tubes. The apparatus should be used in a dark room, but I have obtained quite good results in daylight by fixing a long tube of cardboard with one end around the windows (*A*), and applying the eye to the other end.

Estimation of yeast suspensions in coloured and colourless fluids. In comparing the turbidity of suspensions in colourless fluids I have placed two tubes

of distilled water in the anterior pair of compartments in the comparator, and the tubes containing the suspensions in the posterior pair. This is the arrangement shown in Fig. 1, in which a tube containing a yeast culture is pictured in the left-hand compartment, and a standard in the right. A small degree of colour in the yeast suspension does not interfere with the comparison.

I have had little occasion to estimate suspensions which were coloured to such a degree as might interfere with the estimation. But in the estimation of the most highly coloured suspensions a control tube of the coloured fluid (containing no suspended matter) must be substituted for the tube of water in front of the standard suspension. At the same time a screen must be placed between the source of light and the standard tube to compensate for the cutting off of light from the suspended particles, owing to the colouring matter. This screen should be chosen of such a colour that, on looking down through a tube of distilled water placed over it, and through a control tube of the coloured fluid, the same tint is observed, the tubes being filled to the level of the upper margins of the windows in the front of the comparator. Obviously this procedure does not perfectly control the colour factor, but preliminary experiments have shown that it yields very good results in the estimation of suspensions of yeast in highly coloured fluids.

In all comparisons of turbidity the suspensions were shaken as thoroughly as possible and compared in the apparatus immediately. Growing cultures of the yeast "381" readily form uniform suspensions if the tube is held upright between the thumb and fingers and shaken with a slight rotatory movement. This is especially the case up to the third day of growth. Subsequently, the yeast tends to adhere to the bottom of the tube and some pigment formation commences which renders more difficult the comparison with the barium sulphate standards, particularly in the case of rich development of the yeast. I have frequently observed that up to the third day of growth the cultures contain a very high proportion of single cells, and only a few cell-aggregates containing more than two cells. As growth continues larger aggregates generally predominate, and these tend to adhere to the walls of the tube. By admitting a small glass bead I have been able to obtain suspension of the cultures more easily, and experiments are in progress to determine whether this procedure is injurious to the yeast.

Thus the method is most suitable for the estimation of young growing cultures of yeast and becomes less accurate and convenient as the age of the cultures increases. This does not appear to me to be a great disadvantage as the growth of most cultures is very slow after the 70th hour.

Results.

The method has been applied to the estimation of suspensions of two species of yeast. Results are tabulated below (Table I) and recorded graphically in Fig. 2. They may be considered as calibrations of the barium sulphate suspensions in terms of yeast.

For each suspension of yeast a figure is given in the third column of the table denoting the concentration of cells it contained, and in the fifth column showing the concentration of barium sulphate which was required to display the same turbidity. In cases where the turbidity was not exactly matched by a standard I have judged the concentration of the standard which would have matched it by reference to the two standards nearest to it. In these cases I have shown the figures for turbidity in parentheses.

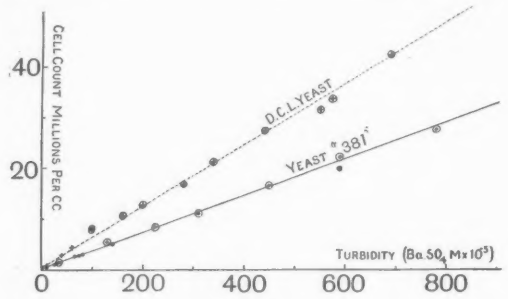

Fig. 2.

The remarks in the fourth column of the table need some explanation. In determining the number of cells present by a count a Bürker chamber was used, four samples were usually counted, and the mean taken. The maximum deviation of the figure for any individual sample from the mean was 21 %, the minimum 1 %, while in most cases the deviation was about 8 %. In the earlier determinations only the large squares of the chamber were counted. Such procedure necessarily limits the concentration of cells which can be determined by a direct count—without dilution—to some value between 0·25 and 5·0 millions per cc. But any suspension containing more than 50,000 cells per cc. possesses turbidity to a degree which can be matched by one of the standard suspensions. Thus in many cases where the turbidity of a yeast suspension was determined its cell content was not actually estimated by a count, but was calculated from the dilution used in preparing it. The remarks in the fourth column of the table refer to the method employed in obtaining the figures recorded in the preceding column. "Observed" values were determined by cell-count, whereas "calculated" values were calculated from the dilution of the suspension which was used.

The counts of suspensions II and III show that the discrepancy involved in diluting suspension II ten times was 14 %. But the counts of the four samples of suspension III showed a deviation of 21 % from the mean, presumably because the concentration of cells was low. In more recent determinations, of which suspensions XXIX and XXX are typical examples, larger concentrations have been counted directly using the small squares in the Bürker chamber. The error in diluting suspension XXIX ten times to

prepare XXX was 4·5 %. Thus when counts have been made of diluted suspensions the results have agreed with the values calculated from the dilution within the limits of experimental error. I therefore feel justified in including in my results those observations in which direct counts were not actually made, but in the graph such observations have been charted with distinguishing marks, *i.e.* enclosed in small circles (⊙ and ⊕).

Table I.

Fresh "D.C.L." Yeast.

Suspension number	Method of preparation	Millions of cells per cc. = C	Observed or calculated	Turbidity in terms of $BaSO_4$ $M10^{-5}$ = T	T/C
I	Suspended in water containing phenol	31·5	Calculated (from mean of II and III)	550	17·46
II	25 cc. I in 250 cc.	2·93	Observed	40	13·66
III	50 cc. II in 500 cc.	0·34	,,	(4)	11·78
IV	25 cc. I in 100 cc.	7·9	Calculated	100	12·66
V	100 cc. II in 500 cc.	0·59	,,	(9)	15·26
VI	Another sample suspended as I	42·2	Calculated (from VII)	(690)	16·37
VII	50 cc. VI in 500 cc.	4·22	Observed	60	14·24
VIII	25 cc. VI in 100 cc.	10·55	Calculated	160	15·18
IX	50 cc. VI in 100 cc.	21·1	,,	(340)	16·12
X	30 cc. VI in 100 cc.	12·66	,,	200	15·80
XI	40 cc. VI in 100 cc.	16·88	,,	(280)	16·60
XII	65 cc. VI in 100 cc.	27·42	,,	(440)	16·08
XIII	80 cc. VI in 100 cc.	33·76	,,	(575)	17·05
XIV	25 cc. XIII in 100 cc.	8·44	,,	100	11·86
					Mean = 15·01

Yeast "381." This is a pure strain of *S. cerevisiae* which I have used throughout a series of studies on yeast growth. "381" is the catalogue number in the National Collection of Type Cultures. Suspension XV was prepared from a 48 hours' growth at 25°, on medium containing cane sugar, salts and yeast extract. The cells were thoroughly washed on the centrifuge before use.

XV	Suspended in water containing phenol	27·7	Calculated (from XVI)	(780)	28·16
XVI	5 cc. XV in 50 cc.	2·77	Observed	(75)	27·07
XVII	40 cc. XV in 50 cc.	22·16	Calculated	(590)	26·65
XVIII	30 cc. XV in 50 cc.	16·62	,,	450	27·09
XIX	10 cc. XV in 50 cc.	5·54	,,	(130)	23·46
XX	25 cc. XVI in 50 cc.	1·39	,,	(35)	25·20
XXI	5 cc. XX in 50 cc.	0·14	,,	(3)	21·43
XXII	25 cc. XVII in 50 cc.	11·08	,,	(310)	27·99
XXIII	25 cc. XVIII in 50 cc.	8·31	,,	(225)	27·07
XXIV	Isolated observations on various cultures	0·24	Observed	(8)	33·33
XXV		5·0	,,	(140)	28·00
XXVI		2·91	,,	80	27·50
XXVII		2·68	,,	(65)	24·27
XXVIII		0·96	,,	(27)	28·14
XXIX	Another sample suspended as XV	19·75	,,	(590)	29·96
XXX	5 cc. XXIX in 50 cc.	1·87	,,	60	32·07
					Mean = 27·34

Discussion.

If the figures in the third and fifth columns of the table are plotted on a graph, as is shown in Fig. 2, the points fall on a straight line, the slope of which depends on the species of yeast considered. There is a direct relationship, therefore, between the number of cells present per cc. and the "concentration"

of barium sulphate which is required to give the same turbidity. By determining the latter in the case of a given suspension of yeast its cell-content can be estimated by reference to the graph, or by calculation from the constant T/C. If the lines plotted, or the mean value of the constant T/C, can be considered as representing the true relationship between turbidity and number of cells, and the fact that they are derived from a number of observations seems to justify this assumption, then it will be seen that, for the yeast "381," the maximum error of any single observation is 17 %, while over the greater part of the graph the error is not greater than 3 %. For "D.C.L." yeast the corresponding figures are 21 % and less than 7 %. These figures compare favourably with the average error involved in a direct cell-count. I have not considered the error of the figures for suspensions showing a turbidity less than 10 *m* $BaSO_4$ for reasons already stated.

The graph shows that the method can be used conveniently for the estimation of the concentration of cells in yeast suspensions between 0·5 and 35 millions per cc., in the case of yeast "381." The estimation is less accurate for smaller concentrations, and is not possible if there are less than 40,000 cells per cc. In the case of concentrations greater than 35 millions per cc. the suspension of cells must be diluted to come within the range of the standards.

It would perhaps be simpler to use some suspension other than barium sulphate as a standard, *e.g.* gum mastic. One standard suspension of such a substance could be employed, and the turbidity of the cell suspension determined in terms of the dilution of this standard which would be required to display the same turbidiness. I have not attempted any modifications of this nature, as the method in its present form has quite fulfilled all my requirements. Indeed, I hope that it may be modified for use in the estimation of cells other than yeast, *e.g.* bacteria, blood corpuscles, etc.

Summary.

A method is described for the rapid estimation of the number of cells in suspensions of yeast, based on a comparison of the turbidity of the suspension with that of standard suspensions of barium sulphate.

In conclusion I desire to thank Prof. Peters for his advice and criticism, and the Medical Research Council for a part-time grant.

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LXVIII. ON THE ISOLATION FROM TISSUES OF CERTAIN PENTOSE DERIVATIVES.

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(*Received March 17th, 1927.*)

In a letter to *Nature* [Winter and Smith, 1923, 1] reference was made to a carbohydrate present in rabbits' tissues which appeared to be of an unusual type. At that time it was believed [Dudley and Marrian, 1923] that an animal at the point of insulin convulsions had little or no glycogen present in the tissues, and it was suggested that there might be a conversion of glycogen or glucose into this unidentified sugar under the action of the insulin. The chief point that was noted about this substance was its apparent instability during the process of isolation from the tissues. Extracts which had been kept for a short time in a semi-dry state showed a markedly diminished sugar content as evidenced by the lessened intensity of the Molisch reaction, and in some cases this test became negative. It was also found that boiling for a short time with very weak acid would appreciably restore the property of giving a positive α-naphthol reaction. The more recent experiments to be described have confirmed the truth of these observations. Though the fresh extracts were without action on Fehling's solution, before and after hydrolysis with mineral acid, it had been found that the incubation of such an extract with fresh muscle tissue led to an increase in the amount of reducing sugar, suitable control experiments being performed. This indicated that there was in the extracts a carbohydrate which was potentially a reducing sugar, yet combined in such a way that the reducing power was masked; and that hydrolysis with acid was not capable, or had been carried out under such conditions that it was not capable, of liberating the reducing sugar. It is only as a result of later work with the methylated substance that it has been possible to demonstrate definitely the reducing nature of the sugar. At that time there was great difficulty in attempting to isolate such a sugar, since there was only the α-naphthol reaction that could be used as a guide during the many operations. It was suggested by Berkeley [1923] that the unknown substance was of a pentose nature. Reasons were given [Winter and Smith, 1923, 2] for thinking that this was incorrect, since the extracts used were not sufficiently fresh to give more than minimal amounts of furfural. Later, when it had been recognised that the estimations must be carried out within a short time after extraction, it was found

that considerable quantities of furfural were formed on distillation with 12 % HCl, and since then this has been used as the method for checking the different fractions during the process of isolation. The work has been carried on continuously during the past three years, and although it is still incomplete, it must be interrupted for a time. The following is an account of the results which have so far been obtained.

Experimental.

In working with the liver and muscle of rabbits, alcoholic extraction was used. When it was found necessary to work on a large scale, the quantity of fat removed by the alcohol made the subsequent operations very difficult. The use of acetone is a great improvement in this respect. Normal goats were used as the source of fresh liver and muscle. Determinations of the pentose content (unpublished experiments with W. Smith) of the tissues of normal and insulinised animals did not show sufficient increase in the latter to warrant the use of large amounts of insulin, and in many cases the results were inconclusive. It was soon recognised that if the sugar was as unstable as the preliminary experiments had indicated, the greatest chance of success in isolation of the substance lay in stabilising it at an early stage after extraction. A means of alkylating the sugar was therefore looked for. To carry it out effectively it was desirable to precipitate the pentose sugar, as adsorption compound or otherwise, in order to obtain it relatively free from other substances. Though the precipitant adopted, silver, only carries down a part of the desired sugar, to methylate sugar contained in such a large syrupy mass as results from concentration of the acetone extract of material from only one goat was obviously out of the question; and the alternative of attempting to remove other constituents of the syrup, leaving the half gram of pentose, was equally impossible, even supposing that the sugar would not have been destroyed in the large number of necessary operations, and the preliminary experiments all tended to suggest that such destruction would take place. It has been found that, though precipitation with silver is effective in removing a considerable amount of pentose, the sugar cannot again be precipitated with this reagent. There is no difference in this respect whether the silver is removed as sulphide or chloride. After removal of hydrogen sulphide or chloride from the filtrate, followed by concentration of the solution *in vacuo*, the re-addition of silver sulphate causes the formation of a precipitate, which does not contain more than a trace of furaloid substance. This fact suggests that the unstable pentose is held as adsorption compound on the original silver precipitate, which probably consists largely of purine material. At this stage the only evidence of the presence of pentose was the formation of furfural on distillation with acid. Glycuronic acid reacts in this manner, and it was essential to be sure that the latter substance was not responsible for the facts observed. The naphthoresorcinol test was usually negative, even when a large amount of pentose was indicated by the quantitative estimation. On some occasions a

slight positive reaction was obtained, but there was no constancy in the results from this test.

No attempt has been made to keep the extracts of liver and muscle separate, since the sole object of the experiments was the isolation of as much material as possible. It has been found impossible to work separately with the liver of a goat, since the large amount of reducing sugar which is formed in the short time before removal of the organ causes rapid reduction of the silver solution. When the liver is minced and mixed with the muscle, the combined extract can be dealt with without difficulty, and it has therefore been possible to infer that the liver brings to the silver precipitate approximately the same amount of pentose as does the whole of the muscle. Two goats are usually used. They are pithed, bled and skinned; the livers and as much muscle as possible are removed, minced, and placed in cold commercial acetone in the proportion 1·25 l. per kg. of fresh tissue (about 14 kg.). The mixture is allowed to stand with occasional shaking for two days at room temperature. The liquid is then strained off and filtered, and as much acetone as possible removed from the tissue by means of a press. The acetone is then distilled off, and the watery extract concentrated under reduced pressure at 40° until the volume is about 500 cc., the concentration being completed about nine hours after the commencement of filtering. The thin syrup is thoroughly cooled, and treated with saturated baryta (cold) until no further precipitation occurs; this point is readily observed with experience owing to the flocking of the precipitate which takes place. The fluid is then centrifuged, and the clear solution poured off. The precipitate is stirred up very thoroughly with cold water. After centrifuging, the washings are added to the main bulk, and the whole neutralised with dilute sulphuric acid. Using a large machine of 1200 cc. capacity, the liquid need not be alkaline for more than 30 minutes; it is unlikely that in this time the alkalinity will have done more than bring about equilibrium between stereoisomers, certainly no appreciable resinification of sugars occurs. It has not been found possible to avoid the use of baryta. The barium sulphate is not removed from the neutralised fluid, but excess of silver sulphate is added. Approximately 6 l. of the saturated solution are required for about 14 kg. tissue, and when the precipitate flocks and begins to settle appreciably within two or three minutes after the last addition, the right point is reached. The mixture is then placed in the dark for an hour, and as much as possible of the clear fluid is siphoned off. The remainder, usually about 3 l., is centrifuged. It is important to protect the material from the action of light at this stage. The solid in each cup is stirred up with 95 % alcohol, and again spun down. Then follow two washings with absolute alcohol, and two with dry ether. It was originally decided to methylate the sugar contained in the silver precipitate with methyl iodide and silver oxide, as in the methods developed by Purdie and Irvine; the last ether washing was therefore poured off, and the solid transferred to a flask. 100 cc. dry methyl alcohol, and 50 g. methyl iodide were added, and the mixture was heated at 50–60° for two hours.

After the addition of 10 g. silver oxide, and 10 g. methyl iodide, heating was maintained for a further four hours. The silver was filtered off and washed with methyl alcohol; the filtrate was evaporated to dryness, and heated at 70–80° *in vacuo* overnight. Next day about 20 cc. cold methyl alcohol were necessary to dissolve the syrup; 20 g. methyl iodide were added, followed by 10 g. silver oxide, in small amounts. For the third methylation 20 g. methyl iodide were used, and the syrup was partly soluble in this medium, only 2 cc. methyl alcohol being necessary to take the whole into solution. 16 g. silver oxide were added slowly, followed later by 20 g. methyl iodide, and 8 g. silver oxide. Two further alkylations were performed using methyl iodide alone. The syrup finally obtained was distilled at 13 mm. pressure. The first fraction began to come over at 110°, and the temperature continued to rise to 180°, with a bath-temperature that was gradually increased to 240°. Three fractions were collected. Considerable charring took place during distillation. The distillates were soluble in water and ether, and gave a Molisch reaction. After hydrolysis with acid a reducing substance was present in the solution. It is remarkable that the distillates all contained organic phosphorus, the amount increasing with the boiling-point. This result was confirmed in all cases in which the methylation was carried out in the above manner. Though the nature of the phosphorus-containing substance in the distillates has not been established, it is very probable that it is derived from a pentose-phosphoric acid compound, such as inosinic acid which is very likely to be present in the silver precipitate. The phosphorus is only slowly liberated on hydrolysis with acid.

0·3 g. of the fraction boiling between 115° and 130° was heated on the boiling water-bath with 4 cc. of *N* sulphuric acid.

Time (mins.)	0	10	20	65
α	+ 1·72°	+ 1·12°	+ 1·15°	+ 1·15°

The acid was removed as barium sulphate, and the filtrate was concentrated to dryness. The syrup was extracted with ether, and distilled after removal of the solvent. The distillate reduced Fehling's solution vigorously, and contained organic phosphorus in considerable amount. As it was unlikely that the sugar sought was in combination with phosphorus, for the pentose of inosinic acid is stable and not likely to show the same phenomenon of a diminished capacity to form furfural when kept for any length of time, a method of removing the phosphorus compound was desirable. This was found to be very difficult to effect by fractional distillation, and, as none of the distillates showed signs of crystallising, the only solution lay in preventing the phosphorus compound from being methylated at the same time as the other pentose. Preliminary extraction of the silver precipitate with methyl alcohol failed to remove the phosphorus-containing substance, but the use of methyl sulphate, as in Haworth's method of alkylating sugars, proved successful in keeping back the phosphoric acid from the outset. The silver precipitate was suspended in dry ether in a dark bottle and decomposed by passing dry HCl gas through

it. This stage was always reached on the same day as the acetone extract was filtered off. Twelve hours later the ether was removed, and the solid shaken with a further quantity of ether (about 100 cc.). After standing for a further 12 hours the ether was replaced with 75 cc. dry methyl alcohol, and the precipitate allowed to stand in contact with the alcohol for two days at room temperature with frequent shaking. The alcohol was then removed and the precipitate shaken with a further 75 cc. of alcohol. Meanwhile the first extract was being shaken with silver carbonate. The second alcoholic solution was separated after 24 hours, added to the first, and the last traces of acid removed. After this treatment a water extract of the silver residues showed only traces of pentose remaining. The pentose content of the alcohol extract was estimated as a routine procedure by removing a known small amount, distilling with hydrochloric acid, and weighing the precipitate of phloroglucide. The solvent alcohol was first removed on the water-bath. In order to methylate the sugar contained in the alcoholic extract with methyl sulphate it was necessary to remove almost the whole of the alcohol, and, whilst it is possible that some destruction of the furaloid substance might occur during concentration, the test samples were treated in a similar manner and would indicate the amount of pentose which was available at the time of methylation. Up to 0·8 g. pentose has been shown in the extract from two goats; sometimes it has been much less than this. For a considerable time it was customary to keep the methyl alcohol solution, freed from acid, of each pair of animals at 0°, until four such extracts were available, and then to combine them and concentrate for methylation. It was assumed that the pentose would be stable under these conditions. The four combined extracts were then concentrated under reduced pressure, or directly on the water-bath (no apparent difference in the yield being observed in this respect), down to 3–4 cc. The syrup was then transferred to an apparatus fitted with mechanical stirrer and dropping funnels, and treated with methyl sulphate and sodium hydroxide. A very vigorous reaction ensued, and the temperature was not allowed to rise above 50° until half the necessary quantity of the reagents had been added, in successive small amounts. For this amount of extract 30 cc. methyl sulphate and 48 cc. sodium hydroxide solution (40 g. in 85 cc. water) were used. The whole of the reagents having been added, the bath-temperature was raised to 70° and maintained at this level for an hour. After boiling for half an hour, the contents were cooled, and extracted twice with chloroform. The chloroform extracts were dried by standing over anhydrous magnesium sulphate for several days, filtered into a flask and the solvent removed. Methylation was then continued with methyl iodide and silver oxide, the whole of the syrup being soluble in the iodide. After heating for six hours, the silver residues were extracted with ether; the solution was dried, the solvent removed, and the syrup distilled under 13 mm. pressure. 0·6 g. was collected boiling between 125° and 131° and, on changing the receiver, no further distillate was collected when the temperature of the bath was raised. A considerable amount of non-volatile

residue remained in the flask. The distillate was neutral and entirely free from phosphorus, while an intense Molisch reaction was obtained. After heating a sample with *N* sulphuric acid at 100° for an hour, the solution reduced Fehling's solution. The next four pairs which were dealt with in a similar manner gave a very poor yield of the methylated derivative, only 0·2 g. being obtained. The initial pentose content of the two batches as deduced from the test sample taken from each alcohol extract was very similar, and it became evident that there was some secondary change proceeding which must account for the wide variation in the yield.

Examination of the alcoholic solution before methylation.

As evidence was accumulating as a result of the alkylation experiments that the pentose substance when methylated was capable of reducing an alkaline copper solution after hydrolysis, it seemed desirable to test the solution in methyl alcohol for the presence of a potentially reducing sugar, and to ascertain whether the process of methylation was after all necessary for the isolation of the pentose. It was possible that a reducing sugar might have been liberated during the decomposition of the silver precipitate with HCl in ether, which would condense with the solvent during the extraction with methyl alcohol under the influence of the HCl which was present before removal of the acid with silver carbonate. An extract was used which showed a pentose content of 0·51 g. The alcohol was removed under reduced pressure, and the residue dried in a desiccator for one day. The syrupy mass was then extracted with cold methyl alcohol (30 cc.). A white solid was left which on examination was found to be hypoxanthine. No pentose residue was found insoluble in cold methyl alcohol. The solution was cleared with charcoal in the cold, filtered, and evaporated again in the desiccator. More solid separated and was removed by extraction as before with alcohol, the syrup going into solution very readily. A test sample showed a pentose content of only 0·170 g., and since the residues had been quantitatively examined each time for pentose with negative results, it must be concluded that decomposition of the desired substance had taken place or some alteration which prevented the formation of furfural on distillation with acid. It was clear, therefore, that if the pentose underwent such a profound change during the mild conditions of the above experiments, it was improbable that the sugar could be isolated without recourse to the stabilisation conferred by methylation. The solution was again evaporated; three weeks later the pentose content had not appreciably altered. It is likely that the residual pentose was ribose, and in combination with phosphoric acid and hypoxanthine. There was organic phosphorus present, and hypoxanthine had also been separated from the mixture. Since silver precipitates purines, it was to be expected that such substances would be present. The syrup was taken up in *N* sulphuric acid, and heated at 100°. There was a steady fall in the polarimeter readings, and a very slight reduction of Fehling's solution was observed.

Time (mins.)	0	10	20	30	90
α	− 0·40°	− 0·31°	− 0·19°	− 0·17°	−0·15°

The silver precipitate from another goat was dried with alcohol and ether and decomposed in the usual manner. During the second extraction with ether, the HCl was removed with silver carbonate. The precipitate was shaken for one hour with a small amount of methyl alcohol, and then filtered. To a part of the clear solution was added some HCl in dry methyl alcohol so that the concentration of acid was 1 %. The optical activity was observed at once, and at varying intervals during the course of 24 hours, but was unaltered. No apparent change was taking place in the carbohydrate under the action of the acid and alcohol, at room temperature. The methyl alcohol extracts are laevo-rotatory, and the methylated derivative finally obtained is dextro-rotatory, the positive value due to the sugar being evidently masked by a large amount of impurity; yet any change in the sugar due to condensation with the solvent should be observed in a change in the optical activity of the mixture. A silver precipitate was dried with alcohol and ether, and then extracted for 5 minutes with methyl alcohol containing 2 % HCl. The solution was centrifuged off and examined, but no change in the rotation was observed on standing. The result was therefore the same whether the sugar was subjected to the action of hydrochloric acid in ether or in methyl alcohol. No evidence was obtained of condensation of the sugar with the alcohol, and if it does proceed it must be completed very rapidly. It was impossible to examine the solution immediately, since a short time must be allowed for some extraction of the precipitate, and the solution has then to be cleared by filtering or by the centrifuge.

The instability of the pentose in the alcoholic extract led to a reconsideration of the advisability of leaving the extract from each pair of animals in cold storage until three or four such solutions were available. It was not possible to deal with more than two goats in one week, the extraction with methyl alcohol not being complete until the evening of the 7th day after the animals were killed. By the end of 3 or 4 weeks the first sample might have a considerably diminished pentose content, even though the mixture was maintained at 0°. This was found to be the case, though the difference was not large as determined by the furfural estimation. When the distillation of the final methylated derivative came to be carried out, however, the difference was remarkable, both qualitatively and quantitatively. While only traces had before been obtained of distillate boiling under 124–125°, now the main fraction was over when this temperature was reached; also, provided that a reasonable amount of pentose had been indicated in the small test samples, a proportional yield of the final product was obtained. In the previous series of experiments there was no certainty that this would be so. Each methyl alcohol extract is now methylated with Haworth's reagents as soon as possible. Directly the acid has been removed, the solution is filtered and concentrated *in vacuo* to a small volume, and treated with 20 cc. methyl sulphate and 32 cc. sodium hydroxide. The united chloroform extracts are kept in the cold store

over anhydrous magnesium sulphate until a number are available. Though not fully methylated, the sugar must be sufficiently stabilised by this time, and there is no advantage in carrying out the alkylation with Purdie's reagents on individual extracts. Three such chloroform extracts were concentrated under reduced pressure, and on the addition of 25 g. methyl iodide the whole of the syrup went into solution. 10 g. silver oxide were added gradually, and the mixture was heated at 55–60° for 7 hours. The silver residues were extracted three times with ether. The ether extract was dried, and some weeks later combined with that obtained in a similar manner from three other pairs of goats. The solvent was removed, and the residue distilled at the water-pump. 1·5 g. of a colourless liquid was collected, boiling between 118° and 122° (fraction 1). The remainder (0·7 g.) distilled at 125–135° (fraction 2). There was a dark viscous syrup left in the flask, which was extracted with ether. Several of these extracts have since been combined, and the contained syrup distilled under a high vacuum. A crystalline fraction was obtained, but these distillates have not yet been examined. The fractions referred to as 1 and 2 were neutral, soluble in water and ether, and gave an intense Molisch reaction. Nitrogen and phosphorus were absent. Kept for a year they have shown no sign of crystallising. Fraction 1 showed $[\alpha]_{\text{Hg green}} = +10{\cdot}85°$ in water, $c = 1{\cdot}94$. OMe = 53·51 %. Hydrolysis was not effected by boiling with $N/100$ acid. $N/10$ sulphuric acid slowly liberated reducing sugar. The following observations were made on a very dilute solution (58 mg. in 4 cc. $N/10$ acid).

$\alpha =$	+ 0·14°	0·16°	0·16°	0·16°	0·14°	0·25°	0·24°	0·24°
Time (hours)	0	½	1	3	6	12	20	28

With normal acid, hydrolysis was rapid, as will be shown later. Fraction 2 showed $[\alpha]_{\text{Hg green}} = +19{\cdot}00°$ in water, $c = 1{\cdot}53$. OMe = 52·52 %. This fraction was more readily hydrolysed with $N/10$ acid, and the change in the polarimeter readings is in the opposite direction to that shown by fraction 1. The solution contained 45 mg. in 4 cc. acid.

$\alpha =$	+ 0·16°	0·10°	0·08°	0·07°	0·08°	0·09°
Time (hours)	0	½	1	1½	2	2½

At the end of this time the solution reduced Fehling's solution markedly. To undertake the methylation of the small quantities of material that were now being dealt with, it was necessary to devise special apparatus. This was constructed by Messrs Flaig and Sons, 57, Hatton Garden, E.C. It consists of a short boiling tube, $3\frac{1}{2} \times \frac{3}{4}$ ins., ground at the neck to receive a condenser or a bent tube. The boiling tube is slightly expanded at the top, so that when the condenser or distilling tube is fitted into the ground joint, mercury can be poured on to the joint to act as a seal. The condenser is of the double surface type, and measures only $5\frac{1}{2} \times \frac{7}{8}$ ins. Fraction 1 was transferred to the boiling tube and methylated with 13·5 g. methyl iodide, and 9 g. silver oxide for 7 hours at 50–55°. The silver residues were extracted with ether. The solution was dried and filtered. After removal of the ether, which is accompanied by

unavoidable loss on account of the volatility of the methylated substance, the syrup was distilled at 13 mm. pressure. The distillate was remethylated, using the same quantities of the reagents as before, and again distilled. The distillate was collected in three fractions. The main (middle) fraction was analysed[1].

3·505 mg. gave 15·552 mg. AgI: 56·00 % OMe.
3·599 mg. gave 15·145 mg. AgI: 55·72 % OMe.
0·0780 g. gave 0·1481 g. CO_2, 0·0615 g. H_2O: C, 51·78; H, 8·76.
0·0877 g. gave 0·1669 g. CO_2, 0·0700 g. H_2O: C, 51·85; H, 8·86.
$C_5H_6O(OCH_3)_4$ requires C, 52·42; H, 8·74.

A cryoscopic determination of the molecular weight in water gave a value of 211. The specific rotation in water for mercury green light was + 17·6° ($c = 1·81$).

0·6 g. of the methylated derivative obtained in a similar manner from eight animals was hydrolysed with 20 cc. of *N* sulphuric acid at 100°.

Time (hours)	0	½	1¼	2¼
$\alpha =$	+ 0·49°	0·53°	0·70°	0·70°
$[\alpha]_{\text{Hg green}} =$	+ 16·33°	17·66°	23·33°	23·33°

The acid was removed with barium carbonate, and the solution filtered and concentrated under reduced pressure. The residue was extracted with absolute ethyl alcohol, and the solvent removed by distillation. A viscid acid syrup was left and decomposition occurred on attempting to distil it at 13 mm. pressure. Only a drop of distillate was obtained which was dextro-rotatory, gave the Molisch reaction, and reduced Fehling's solution. This failure to separate the reducing sugar from the products of hydrolysis was not unexpected in view of the results obtained by Carruthers and Hirst [1922] with trimethyl-methyl-xyloside. Apparently it is necessary to start with the pure α- or β-compound; when a mixture is present a large proportion of breakdown products is formed during the heating with acid. Though in the above experiment the acid used was as weak as possible, and the hydrolysis was stopped as soon as the rotation had ceased to alter, it is possible that the failure to isolate the reducing sugar was due to the presence of two stereo-isomers in the original liquid.

The nature of the parent sugar and of the methylated derivative is as yet uncertain. That the latter is a stable type is clear from the strength of acid necessary to bring about hydrolysis. It is hydrolysed at a similar rate to trimethyl-β-methyl-arabinoside. The following figures were obtained with a solution of the latter compound in *N* sulphuric acid.

Time (hours)	0	½	1¼	2¾
$\alpha =$	+ 0·24°	0·31°	0·35°	0·34°
$[\alpha]_{\text{Hg green}} =$	+ 52·9°	68·5°	77·8°	74·9°

The hydrolysis is complete in the same time in both cases. Though the rather low results obtained in the methoxyl determinations suggest that the substance

[1] Methoxyl determinations were made by the Pregl micro-Zeisl method; for valuable advice on this and other analyses I am indebted to Mr H. I. Coombs. The values for C and H were determined by Mr F. C. Hall, of the Dyson Perrins Laboratory, Oxford.

is not completely methylated, this is not sufficient to account for the large difference between the specific rotations of the unknown sugar and trimethyl-methyl-arabinoside. Xylose can be excluded at once. Methylated derivatives of lyxose and ribose have apparently not been prepared. Even if future work results in the identification of the methylated sugar from the silver precipitate with an alkylated riboside or lyxoside, there remains the problem of the labile nature of the sugar before methylation.

Isolation of a derivative of d-*ribose.*

The ether extracts of the silver precipitates were examined for pentose at an early stage of the work, and it was found that a small amount of furfural was obtained on distilling the residue after evaporation of the ether. When such a syrup was kept for some months there was found to be no disappearance of pentose as occurred in the methyl alcohol extract. It seemed possible, therefore, that the ether had extracted a small amount of another pentose derivative. The substance was finally isolated from each extract as follows. The acid having been removed from the ether with silver carbonate, the solution was dried with potassium carbonate, followed by sodium sulphate. The filtered solution was distilled slowly through a fractionating column, until the volume of ether was reduced to about 10 cc. 50 cc. of light petroleum were added, and distillation was continued. It is important that the petroleum should have previously been distilled. Only that fraction is used which will come through the column on the boiling water-bath. When the flask contains only 5–10 cc. of fluid, it is removed from the bath, and the liquid at once poured out while still hot. On cooling, fat begins to separate. In the flask remains a very small amount of a semi-solid yellow substance from which, however, nearly the whole of the fat has been removed by the treatment with petroleum. The residue in the flask is taken up in a few cc. of cold absolute ethyl alcohol, in which it is very soluble, and filtered. The solution is evaporated in a desiccator, and the resulting syrup crystallises readily. The crystals are dissolved in water, and the solution is cleared with charcoal to remove the last traces of fatty matter. After evaporating to dryness in a desiccator, the substance is recrystallised from absolute alcohol. The slightly hygroscopic compound was dried *in vacuo* over phosphorus pentoxide until the weight was constant. The pentose content was estimated in the usual manner.

9·6 mg. gave 6·6 mg. phloroglucide. 8·0 mg. gave 5·2 mg. phloroglucide. Using for comparison a pure sample of β-methyl-arabinoside, 10·2 mg. gave 6·9 mg. phloroglucide.

The substance contains only carbon, hydrogen, and oxygen; it does not reduce Fehling's solution until after hydrolysis with acid. Heated in a capillary tube, it begins to soften at 58°, and melts at 65–66° (uncorr.). The specific rotation was determined in absolute alcohol. 0·1634 g. in 4·926 g. alcohol gave $\alpha = -3{\cdot}61°$ ($c = 3{\cdot}31$, $l = 1$). $[\alpha]_{\text{Hg green}} = -108{\cdot}8°$. The solubility in water and alcohol and the liberation of reducing sugar on hydrolysis suggest that

the substance is an alkyl-pentoside. Owing to the hygroscopic nature of the compound accurate determination of methoxyl was not possible; a value of 12·27 % OMe was obtained, an approximation which gives support to the suggested nature of the substance. The first step in the identification of the pentose was the preparation of the phenylosazone. A small amount of the pentoside was hydrolysed with 2 % sulphuric acid for 1½ hours on the boiling water-bath. Phenylhydrazine hydrochloride and sodium acetate were added, and the mixture heated at 100° for 20 minutes. The crystals deposited on cooling were recrystallised from dilute pyridine. The specific rotation for mercury green was − 70·2°, Neuberg's pyridine-alcohol mixture being used. The crystalline form of the osazone was identical with that prepared from a sample of arabinose, and the sugar is therefore *d*-arabinose or *d*-ribose. A sample was then hydrolysed, and the optical changes recorded. 0·1408 g. in 10·27 g. 2 % sulphuric acid was heated under reflux on the boiling water-bath.

Time (mins.)	0	45	65	85	135
α =	− 1·19°	0·56°	0·40°	0·27°	0·29°
$[\alpha]_{Hg\ green}$ =	− 86·8°	40·9°	29·4°	19·7°	21·2°

The final specific rotation is very nearly that of *d*-ribose ($[\alpha]_D$ − 19·0), and the identity of the sugar is established. It is uncertain whether the crystalline substance is a methyl- or ethyl-riboside, for there was only a small quantity available for investigation. The yield of the pure substance is only about 2·5 mg. per kg. fresh tissue. If the riboside does not pre-exist as such in the tissues, but is formed from ribose set free from inosinic or guanylic acids during the extraction with ether, it seems likely that it will be the ethyl compound due to reaction with traces of ethyl alcohol, persisting from the operation of drying the silver precipitate, under the catalytic influence of the hydrochloric acid. On the other hand, it is to be expected that a mixture of the α- and β-forms would be produced under these conditions, and the complete crystallisation which takes place when the solution in absolute alcohol is evaporated suggests that only one isomer is present, from the optical activity probably the α-riboside. The curve of polarimeter readings on hydrolysis is regular and indicates the absence of the β-compound, assuming the difference usually found in the rates of hydrolysis of α- and β-sugar derivatives. There is no evidence then for the existence of both isomers in the crystalline material obtained, and it might be that the more soluble form was left behind in the light petroleum used for precipitating the substance. Examination of this solution, however, showed no trace of pentose. Using arabinose, no formation of an ethyl-arabinoside was observed when a suspension of the sugar was treated in a similar manner to the silver precipitate. 200 mg. arabinose were shaken with 200 cc. dry ether, containing 2 cc. absolute alcohol, and 2 % HCl, for 24 hours. The sugar was apparently unchanged, and no weighable residue was obtained on evaporating the ether after removal of acid. It seems unlikely, therefore, that the riboside which has been isolated is artificially produced from nuclear material during the processes used for its extraction, and

it is probable that it is present in the fresh tissues. The pentose which was isolated in the form of its methylated derivative is present in relatively large amount as compared with the alkyl-riboside, approximately 0·005 g. as compared with 0·0002 g. per 100 g. fresh tissue. Actually, however, the quantity is very small, and this fact in conjunction with the marked lability of the compound renders it probable that the sugar is an intermediate in carbohydrate metabolism.

SUMMARY.

Results are given of an investigation of two pentose derivatives present in animal tissues. One contains *d*-ribose, and is probably an alkyl-riboside. The other is unstable, and only the methylated derivative has been isolated. Its nature has not been determined.

I wish to thank Sir F. G. Hopkins for his continued interest throughout the work and for much expensive material used. For the year 1925 the remaining expenses were partly defrayed by a grant from the Government Grant Committee of the Royal Society. This research was contained in reports to the Trustees of the Beit Memorial Fellowships for Medical Research in the years 1924–26.

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LXIX. THE ETHER-SOLUBLE SUBSTANCES OF CABBAGE LEAF CYTOPLASM.

III. THE FATTY ACIDS.

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A METHOD of preparing the ether extract of leaf cell cytoplasm in bulk from green unheaded cabbage (*Brassica oleracea*) has been described in a previous paper [Chibnall and Channon, 1927, 1]. A table showing some of the fat constants of the material was given, and it was pointed out that the addition of 4 volumes of acetone to the ether extract results in the precipitation of about 40 % of the material. The chemical nature of certain of the constituents of this precipitate has already been discussed [Chibnall and Channon, 1927, 2] and further investigations of the remaining substances present in it are in progress. The fraction of the extract which is not precipitated from ether by acetone—some 60 % of the whole—contains the fatty acids which are not in complex combination in phosphorus-containing substances. It is with these fatty acids that this communication deals.

On evaporation of the ether-acetone extract *in vacuo*, there is obtained a hard, waxy mass which is almost black in colour on account of the presence in it of chlorophyll. It would obviously have been desirable to remove the pigment before proceeding further, but as this was impossible without the loss or chemical alteration of some of the other substances present, it was necessary to use this pigmented material without further treatment.

Preparation of the fatty acids and the unsaponifiable matter.

After removal of the ether and acetone *in vacuo*, the material was saponified with excess of strong alcoholic potash for 1 hour, and the alcoholic solution poured into water. From this aqueous solution the unsaponifiable matter was removed by exhaustive extraction with ether and the ether extract washed with water. The water washings were united to the main bulk of the aqueous soap solution. On evaporation of the ether solution, there remained a hard orange brown mass which was re-saponified with 2 *N* sodium ethylate, and then extracted as before. The aqueous solution and the washings from the

ether extract were united to the main bulk of the soap solution obtained during the initial saponification. On removal of the ether, there was obtained the unsaponifiable fraction, the nature of which will be discussed in a subsequent communication.

The aqueous solution, after the removal of the unsaponifiable matter, will contain not only the soaps produced by the saponification of all the esters present in the free fat, but also the alkali salts of the acids produced by the degradation of the chlorophyll originally present. On acidification of the soap solution therefore, there will be precipitated not only the fatty acids but also the chlorophyll derivatives, which Willstätter's researches [Willstätter and Stoll, 1913] indicate will be phytochlorin *e*, $C_{32}H_{32}ON_4(COOH)_2$ and phytorhodin *g*, $C_{32}H_{30}O_2N_4(COOH)_2$. Ether extraction of the soap solution removes the fatty acids and these "chlorophyll" acids. After the usual treatment of the ether extract, there remains behind a black fatty mass which can be fractionated into products soluble and insoluble in light petroleum.

The former are the fatty acids whilst the latter are the chlorophyll derivatives with, possibly, hydroxylated acids. The following table illustrates the general nature of the unsaponifiable matter and the fatty acids, obtained by the foregoing methods from the ether-acetone filtrate.

Table I.

	Autumn sown (picked April and May)				Spring sown (picked August)	
Batch	*D*	*E*	*F*	*G*	*K*	*M*
% of total ether extract not precipitated by acetone	59·5	58·0	59·0	58·5	56·0	60·1
Iodine value	141	147	152	143	154	—
Saponification value	151	158	152	—	149	—
% of unsaponifiable matter	16·9	16·5	17·0	17·1	24·1	25·0
% sterol	3·63	2·65	3·70	3·61	3·80	3·70
Iodine value	113	117	119	122	105	97
% fatty acids	23·2	23·1	23·0	23·6	15·6	16·3
Iodine value	173·5	203·5	201	197	206	—
Neutralisation value	209	206	198	—	197	—

From this table, it will be seen that the general nature of the ether extract in all the batches is very similar save that there appears to be a definite difference in the amounts of the fatty acids and of the unsaponifiable matter in the material obtained during the spring and summer.

Fatty acids soluble in light petroleum.

By evaporation of the solvent, the fatty acids remain as a pale yellow, almost liquid mass, free from chlorophyll degradation products.

Saturated acids. 7·8 g. of batch *H* and 5·7 g. of batch *E*, 13·5 g. of fatty acids in all, having an iodine value of 205, were treated by the method of Levene and Rolf [1922]. By fractionation of the barium soaps in benzene containing 5 % of 95 % alcohol, there were obtained 4·63 g. of more saturated

acids having an iodine value of 163, 7·87 g. of less saturated acids having an iodine value of 218. The result of this treatment of the barium soaps shows that the separation has not been very satisfactory. This is not surprising when it is known that by far the greater bulk of the fatty acids consists of acids of 18 carbon atoms containing 2 and 3 double bonds; the precipitate of the barium soaps of the saturated acids and oleic acid contains, therefore, a considerable amount of the barium soaps of these unsaturated acids. The 4·63 g. of more saturated acids were further fractionated by conversion into the lead soaps and treatment of these soaps with ether. The soaps insoluble in ether yielded 1·45 g. of solid acid, which was separated into three fractions by crystallisation from 95 % alcohol. These fractions weighed 0·38 g., 0·65 g. and 0·34 g. respectively, and they gave the following analyses:

Fraction (1)	0·0854 g.	substance	0·2356 g. CO_2 and 0·0969 g. H_2O
	0·3772 g.	„	required 1·95 cc. $\frac{n}{2}$ *N* KOH for neutralisation
(2)	0·0822 g.	„	0·2266 g. CO_2 and 0·0914 g. H_2O
	0·4802 g.	„	required 2·56 cc. $\frac{n}{2}$ *N* KOH for neutralisation
(3)	0·3402 g.	„	„ 1·79 cc. $\frac{n}{2}$ *N* KOH „

Calculated	$C_{16}H_{32}O_2$: C, 74·92; H, 12·58.	M.W. 256; M.P. 63·4°
	$C_{18}H_{36}O_2$: C, 75·93; H, 12·78.	M.W. 284; M.P. 70–71°
Found. Fraction (1)	C, 75·23; H, 12·60.	M.W. 271; M.P. 54–55°
(2)	C, 75·18; H, 12·43.	M.W. 263; M.P. 54–55°
(3)		M.W. 266

The amount of material available was obviously too small for the definite identification of the saturated acids present, but the results would suggest that they consist of about 70 % palmitic together with 30 % of stearic acid, the melting-point of which mixture is given as 56° [Lewkowitch, 1921]. It will be seen that 13·5 g. of the acids have given 1·45 g. of saturated acid.

Unsaturated acids. The liquid acids generated from the lead soaps soluble in ether were again fractionated by the barium soap-benzene method. The insoluble barium soaps yielded only 0·225 g. of fatty acid having an iodine value of 127. As oleic acid has an iodine value of only 90, it is clear that only a part of this 0·225 g. can be oleic acid; indeed, having regard to the fact that the bulk of the fatty acids are highly unsaturated, it might well be that this 0·225 g. consists not of oleic acid but of saturated acid contaminated by those unsaturated acids.

The 9·2 g. of less saturated acids obtained from the two fractions of the barium soaps soluble in benzene-ether were brominated in anhydrous ether at 0° and were then separated into three fractions: (1) 4·95 g., insoluble in ether, (2) 10·0 g., insoluble in light petroleum, (3) 3·22 g., soluble in light petroleum. These fractions gave the following analyses:

Fraction (1)	0·0879 g.	substance	0·0866 g. CO_2 and 0·0301 g. H_2O
	0·1244 g.	„	required (Stepanoff) 10·1 cc. *N*/10 thiocyanate
(2)	0·4133 g.	„	„ „ 28·1 cc. „ „
(3)	0·1458 g.	„	„ „ 8·2 cc. „ „

Calculated	$C_{18}H_{30}O_2Br_6$; C, 28·49; H, 3·99;	Br, 63·26; M.P. 180–181°
	$C_{18}H_{32}O_2Br_4$	Br, 53·28; M.P. 113–114°
	$C_{18}H_{34}OBr_2$	Br, 36·2
Found. Fraction (1)	C, 28·12; H, 3·88;	Br, 63·02; M.P. 180°
(2)		Br, 54·30
(3)		Br, 44·9

Fraction (2) was debrominated and then hydrogenated with platinum black by the Fokin-Willstätter method. The saturated acids so obtained were crystallised twice from 90 % alcohol and four times from acetone, and gave the following analyses:

0·0850 g. substance 0·2362 g. CO_2 and 0·0991 g. H_2O
0·5698 g. „ required 20·2 cc. $N/10$ NaOH for neutralisation
Calculated $C_{18}H_{36}O_2$: C, 75·93; H, 12·78; M.W. 284; M.P. 70–71°
Found C, 75·79; H, 12·95; M.W. 281; M.P. 69–70°

Fraction (1) is clearly hexabromostearic acid, showing that the original fat contained 1·78 g. of linolenic acid. Fraction (2) consists of the tetrabromo-acids of the C_{18} series. Repeated attempts to obtain crystalline tetrabromo-stearic acid from a variety of solvents failed, and it seems probable that there was present in the original fat a doubly unsaturated C_{18} acid which does not yield a solid tetrabromide. The bromine content of fraction (3), namely 44·9 % bromine, suggests that this is a mixture of tetra- and di-bromostearic acids of bromine content 53·28 % and 36·2 % respectively. If this be so, then this fraction contains dibromide corresponding to 1 g. of oleic acid, which did not precipitate when the barium soaps were treated with the benzene-ether mixture, possibly on account of the presence of a preponder-ance of more unsaturated acids; alternatively, all the soaps of the saturated fatty acids may not have been precipitated, and in this case fraction (3) may consist of tetrabromostearic acid contaminated with saturated acids.

Glycerol. It was of interest to see whether glycerol was present in the ether extract in a form other than the combination with fatty acids and phosphorus already described [Chibnall and Channon, 1927, 2]. Accordingly the glycerol content of the acetone-ether extract was determined by the method of Zeisel and Fanto [1903]. The flask containing the material was heated to 112° for 2 hours, by which time the precipitate had settled. The unsaponifiable matter gave a slight precipitate when treated in the same way; 0·5 g. of crystalline chlorophyll gave no precipitate. Accordingly the necessary correction has been made when calculating the ratio of glycerol to fatty acids.

Estimation of glycerol. The acetone-ether-soluble material of batch *G* from autumn sown cabbage contained 39·4 % of fatty acids and 28·7 % of un-saponifiable matter; that from the spring sown cabbage of batch *M* con-tained 26·8 % of fatty acids and 41·1 % of unsaponifiable matter.

0·6030 g. of acetone-ether-soluble material from batch *G*: 0·1380 g. AgI = 0·0540 g. glycerol
0·6600 g. „ „ „ *M*: 0·1148 g. AgI = 0·0449 g. „
0·3080 g. of unsaponifiable matter *G*: 0·0150 g. AgI = 0·0059 g. „
0·8268 g. „ „ *M*: 0·0446 g. AgI = 0·0175 g. „

Found: $\frac{\text{fatty acids}}{\text{glycerol}}$ $\frac{4·7}{1}$, $\frac{4·5}{1}$.

The mono-, di- and tri-glycerides of the C_{18} saturated acids give the ratios 3·1/1, 6·2/1 and 9·3/1. A discussion of the glycerides present in the original ether extract will be held over until the analysis of the fraction which may contain hydroxy-acids is complete.

SUMMARY.

The fatty acids of the cytoplasm of the cabbage leaf have been studied; the greater part consists of the unsaturated acids linolenic and linolic acids; palmitic and stearic acids constitute the saturated acids; the presence of oleic acid has not been proved. The ratio of fatty acids to glycerol is 4·6 : 1.

Part of the expenses of the research described in this and the two preceding papers of the series was defrayed by a grant from the Royal Society. One of us (A. C. C.) is indebted to the Department of Scientific and Industrial Research for a full time grant.

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LXX. OBSERVATIONS ON THE BIOCHEMISTRY OF "MUTTON BIRD" OIL[1].

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Many seabirds, especially those of the petrel family, express their annoyance at the approach of an intruder by ejecting the contents of their stomach. This material is usually of an oily nature and in several different parts of the world advantage has been taken of this to obtain an oil that can be put to various uses. Thus the inhabitants of St Kilda collect the oil of the fulmar petrel and on the coasts of Tasmania and New Zealand a "stomach" oil is obtained from birds of the same family.

"Mutton bird" oil, the subject of this paper, is a mobile, reddish brown oil obtained in New Zealand from the petrel, *Aestrelata lessoni*, which nests on certain small islands off the south coast. When about to leave the nest, the young birds are killed for food by the Maoris, and if suspended head downwards a small quantity of the oil mixed with watery fluid can be collected as it flows from the beak. The oil can also be obtained in the same way from the parent birds, and the opinion is widely held that it is a nutrient fluid secreted by the parent birds and fed to the young. It occurred to us that another explanation might be that the oil represents an indigestible residue of the fatty fish (sprats, shell-fish, etc.), on which the petrel is said to feed during the nesting period.

To test this we were led to study the digestibility of the oil and from that we proceeded to trace as far as we could the fate of the oil during absorption and its behaviour when introduced parenterally. As yet we have not succeeded in unravelling the problems that presented themselves but we think it advisable to publish what we have done.

One of us (C. L. C.) has already published [1921] some work on the chemistry of the oil, and has shown that it has a close resemblance to sperm oil and consists largely of cetyl oleate together with esters of related alcohols and acids. It contains only traces of glycerides and a small amount of cholesterol. When our work began, we were not aware of any paper on the fate of cetyl esters in the body beyond the well-known work of Munk [1891],

[1] This paper was despatched from New Zealand before the receipt in that country of Rosenheim and Webster's paper on the stomach oil of the fulmar petrel [*Biochem. J.* (1927), **21**, 111].—Editor.

who showed that spermaceti (cetyl palmitate) is absorbed and appears in the thoracic duct as palmitin. According to Hutchison [1922] spermaceti was at one time used medicinally as an aid to fattening and was said to be well borne and not difficult to absorb. Purdy [1918] reports that in Tasmania an emulsion of mutton bird oil and almond oil had gained at one time a considerable reputation in the treatment of phthisis.

Thomas and Flaschenberger [1923], in work on the absorption of cetyl alcohol and cetyl acetate in dogs, found that a dog could absorb 4 to 6 g. of cetyl alcohol per day on a daily intake of 20 g. and about the same amount of the ester. They call attention to the difficulty in understanding how the cetyl alcohol, a substance insoluble in water and having such a high melting point (50°), can be absorbed, and they suggest that it may be due to combination with the desoxycholic acid of the bile, which Wieland and Sorge [1916] have shown can unite chemically with various similar substances to form compounds that are water-soluble.

A recent paper by Channon [1926] on the absorption and fate of the unsaturated hydrocarbon "squalene" (the "spinacene" of Chapman) is also of interest in this connection, for with both squalene and cetyl alcohol there is the same difficulty of understanding how absorption can occur.

Digestion *in vitro*.

The oil being of the nature of a fluid wax is not easily emulsified. The best results were obtained with gum acacia as recommended by Lueders and Bergeim [1923]. Toluene was added to inhibit bacterial action. The lipase preparation used was a fresh pig's pancreas, minced and pounded in a mortar with a small quantity of bile, then strained through cheese cloth. When 2 g. of this was added to 10 cc. of mutton bird oil emulsion made slightly alkaline to litmus, the mixture became distinctly acid in 17 hours, and on being made alkaline again developed an acid reaction. After 42 hours, it was made alkaline and extracted with ether. On comparing the weight of this extract with that of the fatty acids obtained from the alkaline residue it was found that 42 % of the original oil had been digested, while a control with olive oil emulsion and the same amount of lipase showed 83 % digestion. In another experiment, at the suggestion of Professor Inglis, we added lithium carbonate to the prepared tubes so as to provide a continuous supply of base to neutralise the fatty acids. In 3 days there were formed 3·416 g. fatty acid from 11·0 g. mutton bird oil, while a control with boiled ferment yielded 0·1 g. fatty acid from 10·5 g. of the same oil. This represents 50 % digested.

We could quote several other experiments but these are enough to show that the mammalian pancreatic lipase can hydrolyse the cetyl esters found in this oil, although the action appears to be slow as compared with that on glycerides. We have not had the opportunity to test the lipase of the bird itself under satisfactory conditions.

Absorption.

Ducks. Unsuccessful attempts, lasting over a week, were made to induce two ducks to feed on a paste made of pollard containing about 5 % of mutton bird oil.

Cats. A uniform diet of minced meat, porridge and milk was used and the oil was administered as an emulsion by stomach tube under light anaesthesia. The faeces were collected in 2 to 3 day periods, and extracted with ether after being finely broken up in water. The ether-soluble material was then saponified and the unsaponifiable fraction extracted with ether. In the second experiment the residue of faeces after the first ether extraction was saponified and again extracted so that we could be tolerably certain of having obtained all the cetyl alcohol excreted. The results are shown in Table I.

Table I. *Faeces* (*cat*).

First experiment:	No.	Weight g.	Total ether soluble g.	Total unsaponifiable g.	Remarks
Preliminary, 4 days	I	38	—	1·55	—
Experimental, 2 „	II	30	7·345	5·435	About 22 g. M.B. oil given
Subsequent, 4 „	III	?	1·945	1·415	—
Second experiment:					
Preliminary, 2 days	I *a*	23	2·69	1·58	—
Experimental, 2 „	II *a*	51	8·11	6·14	About 19 g. M.B. oil given
Subsequent, 3 „	III *a*	39	1·21	0·825	—

It will be noticed that in each experiment there was a large increase in the output of unsaponifiable matter, but the increase is less than the total unsaponifiable fraction (cetyl and other alcohols) of the oil given.

Thus in faeces II, if we subtract from the total unsaponifiable part (5·435 g.) the amount (= 0·77 g.) of such material that would normally be excreted on the diet given during the same time, there remain 4·7 g., whereas the unsaponifiable alcohols in the 22 g. of oil administered amount to 8·4 g.

Similarly, in the more atisfactory second experiment, II *a*, the gross output is 6·14 g. unsaponifiable matter, from which we may subtract 0·8 g. as the normal output, leaving only 5·34 g. as compared to 7·22 g. of unsaponifiable alcohols in the 19 g. of oil given.

In addition, we were convinced at the time that much of the unsaponifiable matter of the experimental periods consisted of cholesterols but unfortunately we were unable, owing to other work, to differentiate between the cholesterols and the alcohols of the mutton bird oil.

Although these two experiments were faulty because of the unnecessarily large doses given, the anaesthesia, and the incomplete analysis, they showed that the oil had no serious effect on the health of the cat and that a certain amount of the cetyl and other alcohols had been absorbed.

Rats. I. Three young rats, kept on a fairly uniform mixed diet, received daily doses of approximately 0·08 g. mutton bird oil administered by a pipette as in vitamin experiments. Three others of the same litter, similarly fed, were given the same amount of olive oil. During 16 days they received in all 3·12 g. of the oils. A sample of 25 g. of faeces from each group was boiled with acid alcohol to break up possible soaps; the alcohol was driven off and the residue extracted with ether. The resulting ether-soluble substances weighed in the mutton bird oil group 2·49 g., in the olive oil group 2·89 g. This material was then saponified and extracted with ether—the unsaponifiable fraction weighed 0·45 g. in the former, and 0·43 g. in the latter, and consisted only of sterols.

II. In this second experiment a similar procedure was adopted but the dose was increased so that in 20 days the same three rats received 11·47 g. mutton bird oil, which should have yielded 4·36 g. cetyl and other alcohols. The total faeces were collected and the unsaponifiable portion carefully examined. It weighed 1·523 g. and the first, second, and third crystallisations yielded crystals melting at 137° and in all probability consisted of cholesterol. No evidence of cetyl alcohol was obtained. The fatty acids of the faeces gave an iodine number of 77·8 as compared to 178 for mutton bird oil acids.

III. From the foregoing results we concluded that a rat is able to dispose of at least 0·2 g. mutton bird oil per day, and a further experiment was made in which the dose was increased to 0·5 g. per day. To three adult rats, a total of 12·33 g. of the oil was given over a period of 8 days. The unsaponifiable matter in this amount of oil would be about 4·7 g., while the unsaponifiable matter in the faeces collected during the 8 days weighed only 0·66 g. and fractional crystallisation and melting point determinations again showed that the bulk of it consisted of sterols. A trace of cetyl alcohol was found, enough to be identified by its melting point but not enough to weigh satisfactorily. The iodine value of the fatty acids of the faeces was 67·4 (Wijs).

In these experiments on rats the practical absence of cetyl alcohol and the low iodine value of the fatty acids indicate a very complete absorption of both components of the mutton bird oil up to approximately 3·3 g. per kg. body weight.

Cetyl alcohol experiment. It occurred to us that it would be interesting to test the absorption of cetyl alcohol by itself under similar conditions. A weighed amount of pure cetyl alcohol was mixed with olive oil to the extent of 15·74 %. When slightly warmed this mixture was sufficiently fluid to be administered by pipette as in the foregoing experiments. Two of the same rats as were used for Exp. III were given daily doses of the mixture over a period of nearly 3 weeks, during which they received in all 2·36 g. cetyl alcohol. The total faeces, collected and treated in the same way as for Exp. III, yielded 0·46 g. unsaponifiable matter from which 0·192 g. cetyl alcohol was recovered by crystallisation and recognised by its melting point, so that 91 % was absorbed, or at least disappeared. As compared to Exp. III, however, the absorption

of cetyl alcohol is not so good as that of the mixed alcohols of mutton bird oil, for in Exp. III the alcohols present in the daily dose of oil amounted to 0·19 g. (38 % of 0·5 g.) and this was almost completely absorbed—while, in the cetyl alcohol experiment, the daily dose was only one-third as much (0·062 g. per rat per day) and yet about 9 % remained unabsorbed.

Fate of the mutton bird oil during and after absorption.

Having satisfied ourselves that the oil can be digested and absorbed, we next proceeded to investigate in what tissues or organs the simple chemical change of cetyl or other higher alcohol into fatty acid or other substance occurs. That it takes place naturally in the petrel cannot be doubted for these alcohols are absent from the body-fat of the bird.

To avoid repetition it may be stated here that the method used in estimating the alcohols was to saponify carefully and completely. The unsaponifiable fraction was then separated from the soaps by shaking with ether in a separating funnel and the constituents estimated by fractional crystallisation, weighing and determination of melting points. A certain amount of loss no doubt occurred, for from a mutton bird oil emulsion of known strength in one test case we recovered only 85 % of the alcohols.

(*a*) *Intestinal epithelium.* From the investigations of Munk on fat absorption it has long been known that cetyl alcohol does not appear in the chyle of the thoracic duct while spermaceti is being absorbed, and the intestinal epithelium has commonly been credited with the ability to transform this and other unusual fats into forms natural to the animal. In our experiments the small intestine of the rabbit with about half an inch of attached mesentery was excised, and, after washing out with Locke's fluid, filled with an emulsion of mutton bird oil of known strength to which had been added a strained suspension of the animal's own mashed pancreas and gall bladder bile. The caecal end of the intestine was then joined to the duodenal end by a very short piece of glass tubing, and the whole was immersed in a bath of warm oxygenated Locke's fluid. For from 4 to 6 hours the intestine continued to show movements, and no doubt a certain amount of circulation of the intestinal contents took place. A control of the same amount of emulsion plus pancreas and bile was kept in a beaker in the same bath.

On examining the intestinal contents and the control we recovered the cetyl alcohol to about the same extent in each, and in one case the ratio of the alcohol to the fatty acid was also examined and found to be unchanged. Four such experiments were done and in no case did we find any evidence that the surviving intestinal epithelium had affected the cetyl alcohol. It may be noted, however, that the amount of digestion may have been small, the leucocytes in the wall would be diminished, and the pumping action of the villi impaired owing to there being no pressure of blood in the blood vessels, so that the experiments did not exactly imitate the natural conditions

of absorption. The method used here was based on that used by Cohnheim [1911] in his experiments on intestinal absorption in fishes.

(*b*) *Parenteral administration.* This was tried both subcutaneously and intraperitoneally. A pregnant cat received 16 g. pure mutton bird oil under the skin of the back in two doses, one 14 days after the other, and both as nearly as possible in the same area. Seven days after the second dose the animal was killed.

At the site of the injection the effect was easily recognised by the presence of a deposit of canary-yellow fatty matter. The subcutaneous tissues were matted together so that the skin did not separate easily. The yellow colour could be traced for a considerable distance headwards and ventrally so that it infiltrated the mammary gland on the side of the injection. Wherever it had penetrated it seemed to have caused increased vascularity, probably inflammatory. The uterus contained five embryo kittens, and in each case there were streaks of the same yellow colour in the foetal membranes. Three samples of fat and subcutaneous tissue were taken for analysis, (I) from the actual site of injection, (II) from coloured fatty tissue near the mammary gland, and (III) normal fat from the opposite side of the body. The results of examining these are given in Table II.

Table II.

	Ether-soluble substance g.	Unsaponifiable matter g.	Fatty acids g.	Iodine value of fatty acids
I	13·234	1·708	10·222	91·4
II	14·560	1·438	8·326	90·5
III	12·280	Traces only	Not taken	63·0

The unsaponifiable matter found in these samples consisted of practically pure cetyl alcohol and the figures indicate that the injected oil that was recovered had undergone little or no change. It had merely become mixed with the fatty tissue. Thus in samples (I) and (II), if a calculation is made of the quantity of fatty acid corresponding to the unsaponified alcohols found (in ratio of 60 : 38, which is the ratio in mutton bird oil), and if the iodine value of the fatty acids of the oil (178) and of the cat's normal fatty acids (63) be applied to these respective amounts, the iodine value of the mixture should be 93 to 94, which corresponds fairly well with the figures found, viz. 90·5 and 91·4. We did not attempt to recover all the oil injected because it had spread so widely. The general distribution of the streaks of yellow oil indicated transportation along the lymphatics. We have no explanation to offer of the well-marked presence of the same material in the foetal membranes.

For intraperitoneal injection we first used a cat. Nine g. of neutralised and sterilised oil were injected, and the animal killed 3 days later. The surfaces of the abdominal organs as well as the omentum and mesentery were found covered irregularly with a greasy, yellow, fibrinous deposit. As far as possible

this was washed out with alcohol followed by ether without extracting the deeper layer of colourless fat such as that surrounding the kidneys. The unsaponified alcohols (cetyl, etc.) recovered corresponded to that present in only 3·7 g. of the oil as against 9·0 g. injected. The iodine value of the fatty acids of the ethereal and alcoholic washings showed a rise as compared to normal.

On opening the thorax two small lymph glands were found in the anterior mediastinum. They were stained yellow like the abdominal organs and this also indicates lymphatic transportation.

In a later experiment we used a litter of young rats, and injected into the peritoneal cavity of each 2 cc. mutton bird oil representing 0·67 g. cetyl and other alcohols. One as a control was killed within a few minutes, and the whole carcase was minced and examined for the alcohols in the usual way (saponification, extraction with ether, etc.). The amount recovered from the control was only 0·35 g. In the two others similarly injected and examined at the end of 4 weeks and 5 weeks we recovered only 0·13 g. and 0·14 g. respectively. This probably indicates that some part of the unsaponifiable material (oleyl alcohol?) underwent metabolic change more readily than the cetyl alcohol which accordingly persisted longer in the body of the rat.

The fate of parenterally introduced fat in general was too large a subject for us to investigate, but our results agree with those of Binet and Verni [1925], for, before we had read the abstract of their paper, we had come to the conclusion that the best explanation of our experimental results was that the oil had excited irritative and mildly inflammatory changes which had led to ingestion of the oil by leucocytes and so to its removal from the site of injection; further, that the oil then underwent changes which led to loss of the characteristics of both parts of the ester. The first (subcutaneous) experiment shows that the esters are removed or changed without dissociation of their components—unlike what may occur in the case of cholesteryl oleate in the alimentary tract [Faust and Tallquist; quoted by Leathes, 1910].

Influence of the liver. In the hope of finding some fermentative action on the part of the liver which might change the alcohols, we made one experiment on freshly killed liver incubated with an emulsion of cetyl alcohol. The alcohol was almost quantitatively recovered from this and from the control of boiled liver.

Vitamin A content.

The widely held opinion that the "stomach oil" plays a special part in the nourishment of the young bird led one of us (J. M.) to test its vitamin A activity, and some of the results have been already published [1926].

In the first set of experiments it was found that 0·04 g. and in some cases 0·02 g. daily was sufficient to produce rapid growth and to cure eye trouble in rats on a basal diet lacking in vitamin A. Later experiments on other samples have not always given the same result. Thus a quantity of oil pro-

cured in May 1925 gave maintenance only in doses of 0·06 g. A sample obtained in December 1925 gave good growth when administered at the same dosage. A sample examined in 1926 gave little or no growth. Such variations in activity are of course common in other cases and may have been due to diet, but we have reason to believe that one of the samples may have been subjected to some "refining" treatment by the Maoris before being delivered to us.

An investigation of the alcohols of mutton bird oil on lines suggested by the work of Drummond and Watson [1922], Fearon [1925], and Drummond, Channon and Coward [1925] has been commenced by one of us (C. L. C.) and his results will be published separately. It was found in general that those samples of oil which gave good results on growth also gave the sulphuric acid (purple) test, the trichloroacetic acid test with the oil in acetic anhydride, and the phosphorus pentoxide test.

On distillation of the unsaponifiable fraction of the oil at low pressure (4 mm.) and a temperature of 180°–200° a fraction consisting of practically pure oleyl alcohol came over, which gave the above colour reactions and which also caused a fair amount of growth in three rats which were being fed on basal diet containing oxidised cod-liver oil (20 hours) as source of vitamin D. These preliminary experiments showed that something came over with the oleyl alcohol, which promotes growth, though in two of the rats the eye symptoms persisted. Thus in a doe rat (*Ag* II, 3) 0·2 g. distillate (oleyl alcohol) daily for 4 days and after an interval of a week, during which the weight began to rise, the same dose repeated daily for 5 days caused ultimately a rise to 115 g. from 92 g., the weight when treatment began.

In another doe (*Ah* IV, 1) treated similarly in the same cage the weight rose from 66 g. to 87 g. and in a third (*Ad* V, 2) from 90 g. to 104 g. with cure of eye symptoms. In the two rats first mentioned the eye symptoms remained stationary.

Origin of the oil.

As stated above (p. 484) we at first thought it possible that the oil might be an indigestible residue of the bird's food. During the nesting period the petrels are believed by some to feed chiefly on the shoals of sprats and other members of the herring family that occur in these waters, by others they are believed to feed chiefly on shell-fish. In either case a residue of fatty material might result from gastric digestion of the food, and it has been pointed out by others that birds in general have a poor supply of lipases in their digestive secretions. Against this view, however, there is the evidence of a great degree of uniformity in the composition of the oil, and the low concentration of cholesterol.

During the course of investigations on the food value of New Zealand fishes we took the opportunity to examine the unsaponifiable part of many of the fish fats, liver oils, and shell-fish fats, on the chance of discovering a

clue to the source of the cetyl alcohol—but without success. The chief unsaponifiable alcohol in these oils is cholesterol.

Another view widely held is that the oil is secreted by the parent birds for the nourishment of the young. Such secretion is, we think, very unlikely. The petrel has no crop such as the pigeon has, and the oil is not accompanied by the protein that one would expect if this were a nutritive fluid like pigeon's milk.

As the research proceeded, a third view has gradually impressed itself on us as more likely than either of these, and that is, that the oil is the excess of tail gland secretion swallowed by the parent bird and more or less accidentally included in the material fed to the young. Possibly, also, the young bird may produce and swallow some of its own tail gland secretion. In support of this we may call attention to the fact that cetyl and octadecyl alcohols have been found to occur up to 40 % in the "fat" extracted from the tail gland of geese [Metzner, 1907]. Being unable at present to obtain adult mutton birds, we have investigated the tail gland of ducks. In these we find it easy by mechanical stimulation to cause oily drops to appear on the feathered apex of the nipple-like projection which forms the outlet for the gland. We procured a number of duck tail glands from a local poultry dealer and tested the ethereal extract of these for vitamin A. Doses of 0·1 g. gave no evidence of its presence, but this result is not conclusive, for all would depend on the food of the ducks. That skin secretions may contain vitamin A has already been shown by Gudjonsson [1926]. During the coming winter we hope to secure evidence on this point from the mutton bird itself.

Summary and conclusions.

The oil obtained from the stomach of the mutton bird is capable of being digested, absorbed and utilised by mammals. It may contain vitamin A in high concentration but the amount present is variable. The cetyl, oleyl and other alcohols of the oil are changed during or after absorption so that they can no longer be detected, but experiments on the action of surviving intestinal wall and liver cells failed to show where the change occurs.

On parenteral injection, subcutaneously and intraperitoneally, there is evidence that no local dissociation of the esters occurs, and the oil appears to be taken up by leucocytes and to travel by the lymphatics.

The view is put forward that the stomach oil of the chick is the tail gland secretion more or less accidentally swallowed.

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LXXI. THE INFLUENCE OF THE FATTY ACIDS AND HYDROXY-ACIDS AND THEIR SALTS ON ALCOHOLIC FERMENTATION BY LIVING YEAST.

PART II. PROPIONIC, BUTYRIC, *ISO*-BUTYRIC, GLYCOLLIC, LACTIC, HYDROXY-*ISO*-BUTYRIC, α- AND β-HYDROXYBUTYRIC ACIDS AND THEIR SODIUM SALTS.

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In his previous paper [1926], the author investigated the rate of fermentation of sugar by living yeast with buffer solutions composed of acetic acid or formic acid and their Na, K and NH_4 salts. These lowest homologues of the fatty acids exert a specific action upon the rate of fermentation and it is suggested that a hyperbolic relation exists between the concentrations of these acids in the media and the rates of fermentation. Their inhibiting effect is not the same, formic acid being 5–8 times as potent as acetic acid in diminishing the rate of fermentation.

As to the inhibiting action of fatty acids upon the rate of fermentation, Bial [1902] considered that this was due to the hydrogen ion; consequently their degree of dissociation would decide the requisite amount of the fatty acids to stop the fermentation. This conclusion was confirmed by Hägglund [1914] with acetic and butyric acids. On the other hand, Johannessohn [1912] pointed out that the inhibiting effect of the fatty acids appeared to be due to the undissociated molecule rather than to the dissociation products. With an acetic acid and Na acetate buffer solution, Euler and Heintze [1919] confirmed Johannessohn's observations. On the other hand, according to Duggan [1885], the degree of disinfecting action of fatty acids upon *B. subtilis* is inversely proportional to their molecular weights.

In the present paper, experiments were at first instituted with buffer solutions composed of higher homologues of fatty acids and their salts, in order to ascertain what influence would be exerted on the rate of fermentation, whether a hyperbolic relation would exist between the concentration of the acids and the rate of fermentation, and which acid would be more potent, the lower homologue or the higher one.

METHOD AND RESULTS.

Full details of the experimental technique were given in the previous paper [Katagiri, 1926]. The principal points are as follows: fermentation was carried out by 2 g. of pressed top yeast with 30 cc. of the various buffer solutions containing 1·5 g. of glucose after saturation with CO_2 and keeping the temperature at 25° in a water-bath. The amount of evolved CO_2 was observed at intervals of 5 minutes. For the rate of fermentation, the average number of cc. of CO_2 evolved in 5 minutes during about 1 hour's fermentation was chosen and its value was corrected for variation in the fermenting power of the yeast by a standard fermentation which was always carried out in presence of a 0·2 *M* acetate solution at p_H 4·7. Each p_H value was determined by the capillator method both at the beginning and at the end of the fermentation, after saturation with CO_2.

The amount of free acid was calculated according to the amount added plus the amount that was produced from the salt in the process of saturation with CO_2. The latter amount of the acid was calculated from the amount of CO_2 evolved by the addition of HCl of suitable strength to a mixture having the same composition as the solution used in the fermentation.

The results obtained with propionic acid, *n*-butyric acid and *iso*-butyric acid are contained in Table I and Fig. 1.

Table I.

Acid	Total concentration Propionate (*M*)	Ratio (salt/acid) 1 : 4	1 : 1	3 : 1	1 : 0
		Concentration of free acid (*M*)			
A. Propionic	0·25	0·200	25	0·062	0·015
	0·2	0·160	0·100	0·050	0·013
	0·1	0·080	0·050	0·025	0·010
	0·05	0·040	0·025	0·012	0·005
	0·025	0·020	0·012	0·006	0·003
		Ratio (salt/acid) 1 : 3	1 : 1	3 : 1	1 : 0
	Butyrate (*M*)		Concentration of free acid (*M*)		
B. Butyric	0·5	0·375	0·250	0·126	0·018
	0·2	0·150	0·100	0·050	0·010
	0·1	0·075	0·050	0·025	0·006
	0·05	0·037	0·025	0·012	0·004
	0·025	0·019	0 012	0·006	0·002
		Ratio (salt/acid) 9 : 11	1 : 1	3 : 1	1 : 0
	iso-Butyrate (*M*)		Concentration of free acid (*M*)		
C. iso-Butyric	0·5	0·275	0·250	0·125	0·019
	0·2	0·110	0·100	0·050	0·011
	0·1	0·055	0·050	0·025	0·006
	0·05	0·027	0·025	0·012	0·005
	0·025	0·013	0·012	0·006	0·001

The curves (Fig. 1) obtained when the rate of fermentation with the buffer solutions composed of propionic acid, *n*-butyric acid and *iso*-butyric acid with their sodium salts is plotted against the p_H, are of much the same character as was observed with formate and acetate buffer solutions in the previous paper. The p_H values did not change by more than 0·3 p_H during the fermentation and the mean values have been taken.

The concentration of the buffer solutions again appears to have a remarkable effect on the rates of fermentation, when these are compared at constant p_H, the rate of fermentation increasing rapidly as the concentration of the buffers diminishes. As in the case of acetate-acetic acid and formate-formic acid buffer solutions, however, the rate of fermentation is controlled by the amount of free acid and is almost independent of the total concentration. This is well brought out in Fig. 2. A hyperbolic relation can again be supposed to exist between the rate of fermentation and the concentration of acids in the medium.

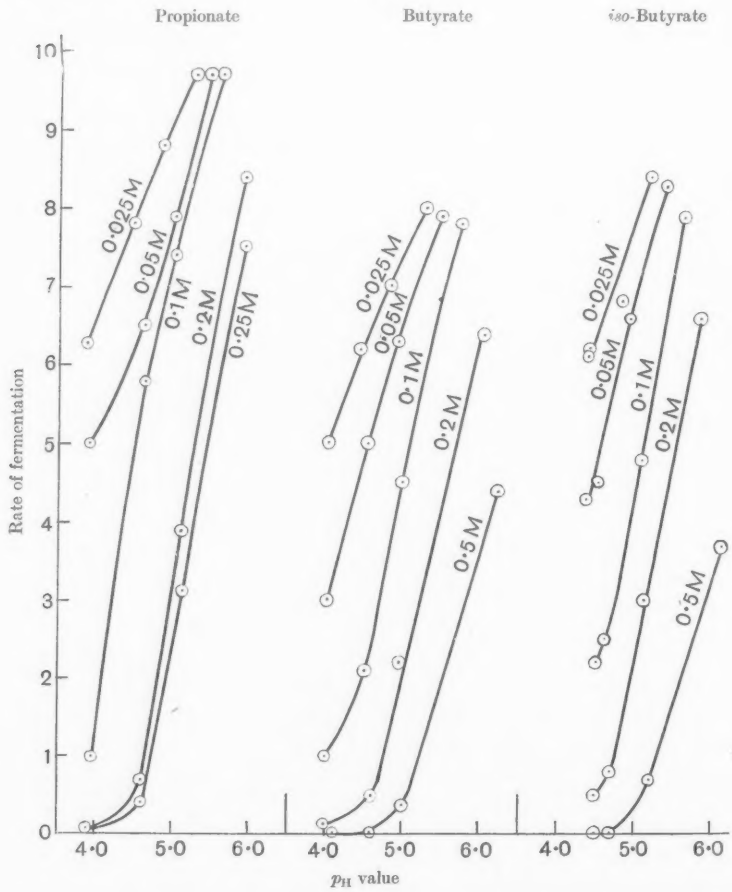

Fig. 1.

For a direct comparison of the inhibiting effects of the various acids, the amount of each acid may be compared in two ways: one in which the rates

of fermentation are diminished to such degrees as 2/3, 1/2, 1/3, 1/4 or 1/10 of the value observed with the least quantity of each acid, and the other in which constant rates of fermentation are observed, *e.g.* 1, 2, 5 and 8 cc. CO_2 per 5 minutes. These values are not constant, but formic acid is relatively more effective at every concentration, while the other fatty acids have nearly the same effect. As examples, the concentrations required to diminish the rate of fermentation to 1/4 and to produce a rate of fermentation of 5 cc. per 5 minutes are given in Table II.

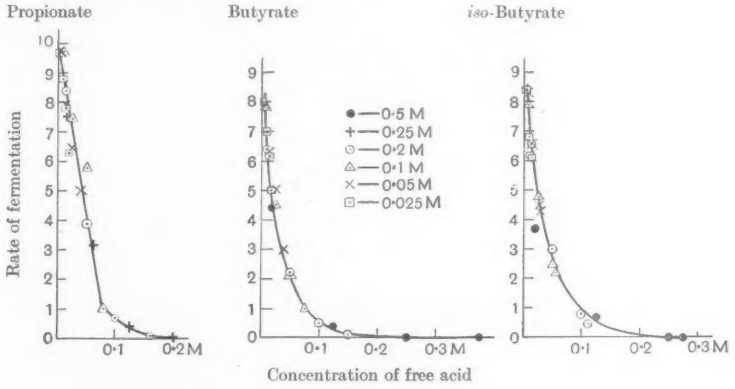

Fig. 2.

Table II.

Acid	Concentration of acid required to diminish the rate of fermentation to 1/4		Concentration of acid with which a rate of 5 cc. per 5 mins. was observed		Concentration of acid required completely to inhibit fermentation				Dissociation constant of the acids (25°)
					Bial [1902]		Johannessohn [1912]		
	M	Relative	*M*	Relative	*M*	Relative	*M*	Relative	
Formic	0·0100	1	0·007	1	0·016	1	0·0139	1	$21{\cdot}4 \times 10^{-5}$
Acetic	0·0500	5·0	0·030	4·3	0·041	2·6	0·0841	6·0	$1{\cdot}86 \times 10^{-5}$
Propionic	0·0675	6·7	0·040	5·7	0·066	4·1	0·1080	7·8	$1{\cdot}4 \times 10^{-5}$
n-Butyric	0·0525	5·2	0·020	2·9	0·050	3·1	0·0926	6·6	$1{\cdot}48 \times 10^{-5}$
iso-Butyric	0·0500	5·0	0·020	2·9	—	—	0·0926	6·6	$1{\cdot}5 \times 10^{-5}$

These numerical relations of the fatty acids agree in general with the results obtained by Bial [1902], Hägglund [1914] and Johannessohn [1912], all of whom carried out the fermentation in absence of a buffer solution.

Vermast [1921] found that the disinfecting action of benzoic acid on *Bacillus coli communis* was controlled by the concentration of the undissociated acid and could be completely accounted for on the basis of the Meyer-Overton partition theory, employing the partition coefficient between water and benzene and basing the calculation on the concentration of undissociated acid.

However, the results obtained at room temperature by Kuriloff [1898] and Georgievics [1913] on the partition of the fatty acids between water and benzene, which are quoted below, show that there is no proportionality be-

tween their partition coefficients and the inhibiting action of the acids on fermentation, so that the relation found by Vermast for disinfection does not hold in the case of fermentation.

Acid	C_1/C_2	$C_1(1-\alpha)/C_2$	Author
Formic	460·5 –340·8	455·7 –339·5	Georgievics
Acetic	55·1 – 80·1	54 – 64	Kuriloff
n-Butyric	0·81– 0·54	0·79– 0·54	Georgievics

where C_1 and C_2 represent the concentration of acid in water and in benzene, respectively, α represents the degree of dissociation of the acid.

Thus, no reliable explanation has yet been put forward as to the effect of the fatty acids in diminishing the rate of fermentation, but it appears that the magnitude of the observed inhibiting effects of the various fatty acids is attributable, in a great measure, to their chemical nature.

Hydroxy-acids.

In order to ascertain the effect of the introduction of a hydroxy-group on the inhibiting properties of the fatty acids on fermentation, experiments were made in the same manner as with fatty acids with glycollic, lactic, α-hydroxy-*n*-butyric, β-hydroxy-*n*-butyric and hydroxy-*iso*-butyric acids and their sodium salts.

The results are given in Fig. 3 and Table III, the mean values of p_H being taken as in Fig. 1.

Table III.

Acid	Total concentration Glycollate (M)	Ratio (salt/acid) 5 : 1	15 : 1	1 : 0
		Concentration of free acid (M)		
A. Glycollic ($K = 1{\cdot}5 \times 10^{-4}$ at 25°)	0·2	0·033	0·012	0·004
	0·1	0·017	0·006	0·002
	0·05	0·008	0·003	0·002
	0·025	0·004	0·001	0·0004

		Ratio (salt/acid)			
	Lactate (M)	2 : 1	5 : 1	15 : 1	1 : 0
		Concentration of free acid (M)			
B. Lactic ($K = 1{\cdot}38 \times 10^{-4}$ at 25°)	0·5	0·167	0·083	0·031	0·002
	0·2	0·067	0·033	0·012	0·002
	0·1	0·033	0·016	0·006	0·002
	0·05	0·016	0·008	0·003	0·0003
	0·025	0·008	0·004	0·001	0·0003

		Ratio (salt/acid)			
	α-Hydroxy-butyrate (M)	3 : 1·02	18 : 3·1	39 : 1·2	1 : 0
		Concentration of free acid (M)			
C. α-Hydroxybutyric ($K = ca.\ 0{\cdot}45 \times 10^{-4}$ at 25°)	0·1	0·025	0·015	0·003	0·001
	0·05	0·012	0·007	0·001	0·0003
	0·025	0·006	0·004	0·0005	0·0003

		Ratio (salt/acid)			
	β-Hydroxy-butyrate (M)	1 : 2	2 : 1	5 : 1	1 : 0
		Concentration of free acid (M)			
D. β-Hydroxybutyric ($K = 0{\cdot}39 \times 10^{-4}$ at 22°)	0·1	0·067	0·033	0·019	0·004
	0·05	0·033	0·016	0·011	0·002
	0·025	0·016	0·008	0·004	0·002

		Ratio (salt/acid)			
	Hydroxy-*iso*-butyrate (M)	2 : 1	5 : 1	15 : 1	1 : 0
		Concentration of free acid (M)			
E. Hydroxy-*iso*-butyric ($K = 1{\cdot}06 \times 10^{-4}$ at 25°)	0·2	0·067	0·033	0·012	0·003
	0·1	0·033	0·016	0·006	0·0009
	0·05	0·016	0·008	0·003	0·0007

When the rates of fermentation are plotted against the p_H (Fig. 3), the picture is totally different from that presented by the unsubstituted fatty acids (Fig. 1). Independently of the total concentration of the buffer solutions,

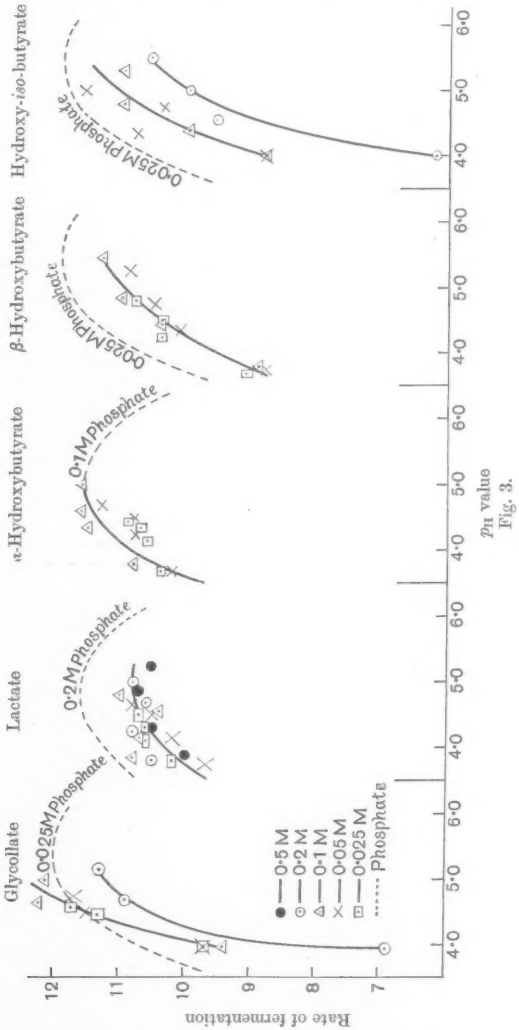

Fig. 3.

the values are found to be situated on regular curves which evidently approach a maximum and closely resemble those given by phosphates (see Fig. 4). The only exception is glycollic acid, which is intermediate in character between

the fatty and hydroxy-acids. These curves show that the rate of fermentation is very largely controlled by the hydrogen ion concentration, but that some other influence modifies this is clear from the fact that the curves are not identical and that the optimum p_H is not the same in each case. This points to some secondary effect due possibly to the specific nature of the acid. This is specially marked in the case of glycollic acid as will be seen from the curves in Fig. 3.

Phosphate buffer solutions.

In order to discuss whether the hydroxy-acids reveal any specific effect on the rate of fermentation, the author's interest was directed towards the inorganic buffer solutions containing phosphate or carbonate, which might serve as standards.

The results with Na phosphate buffer solutions are given in Table IV and Fig. 4. In this figure the p_H values measured at the end of the fermentation were used, since phosphate buffer solutions in the range of about $p_H = 3 - 6$ are not strongly buffered unless the most concentrated solution (0·5 *M*) is used.

Table IV. *Sodium phosphate buffer solutions.*

	Total concentration of phosphate (*M*)	Concentration of H_3PO_4 (*M*)	Concentration of NaH_2PO_4 (*M*)	Corrected rate of fermentation cc. per 5 mins.	p_H Beginning	p_H End
Exp. 34	0·5	0·050	0·450	6·8	2·6	2·6
	0·5	0	0·500	11·6	4·2	4·0
	0·5	0	0·490	11·5	4·9	4·6
	0·5	0	0·479	11·6	5·1	4·9
	0·5	0	0·389	9·3	6·1	6·1
	0·5	0	0·137	4·4	7·0	6·9
Exp. 35	0·2	0·020	0·180	7·0	2·7	2·7
	0·2	0·005	0·195	10·8	3·7	3·4
	0·2	0	0·200	11·1	4·6	3·8
	0·2	0	0·195	11·6	5·1	4·4
	0·2	0	0·163	10·7	6·1	6·1
	0·2	0	0·090	9·4	6·8	6·8
	0·2	0	0·089	7·6	6·9	6·9
Exp. 36	0·1	0·010	0·090	6·9	2·7	2·7
	0·1	0·002	0·098	10·3	3·7	3·5
	0·1	0	0·100	10·1	4·4	3·7
	0·1	0	0·097	11·1	5·1	4·1
	0·1	0	0·085	11·1	6·1	5·9
	0·1	0	0·060	10·1	6·5	6·5
	0·1	0	0·057	9·7	6·8	6·7
Exp. 37	0·025	0·0025	0·0225	7·4	2·8	2·9
	0·025	0·0006	0·0244	9·9	3·7	3·6
	0·025	0	0·025	9·7	4·2	3·7
	0·025	0	0·024	10·0	4·5	3·7
	0·025	0	0·022	11·4	5·7	4·4
	0·025	0	0·018	11·8	6·4	6·1
	0·025	0	0·019	11·8	6·5	6·0
	0·025	0	0·015	10·5	6·6	6·6

When the fermentation rates are plotted against the p_H (Fig. 4), it is seen that the curves for different concentrations of total phosphate are not identical; both the optimum p_H and the sharpness of the maximum vary. The 0·5 *M* curve shows the sharpest maximum, whilst the least sharp is shown by the 0·2 *M* curve.

In the present paper, p_H values are determined colorimetrically; consequently, when p_H values of various concentrations of a buffer solution are compared, a certain correction for the so-called salt error must be taken into

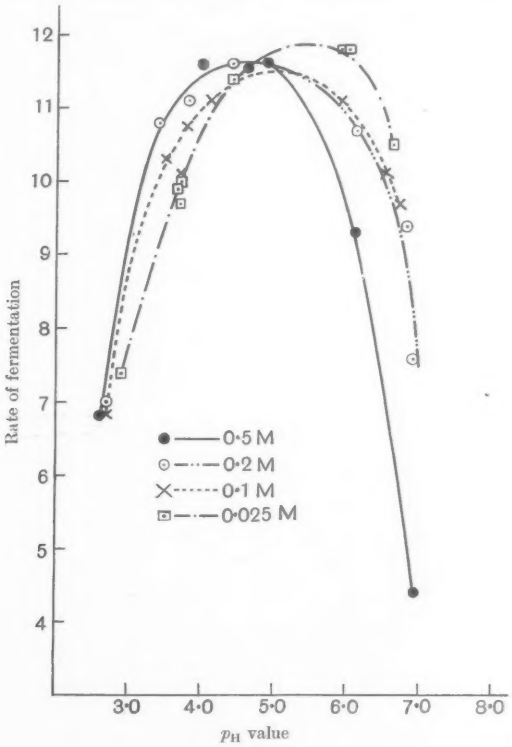

Fig. 4.

account. The correction for salt error becomes greater as the concentration differs from about 0·1 *M* phosphate and the sign of the correction is different on each side of this concentration. In the case of 0·5 *M* or 0·2 *M* solution, the corrected value will be more acidic (p_H less) and in the case of 0·025 *M* the corrected value will be more alkaline (p_H greater). [See Biilmann and Katagiri, 1927.]

From the present uncorrected colorimetric determinations which are tabulated below it is clearly seen that the optimum p_H moves to the alkaline

side as the concentration of the phosphate buffer solution diminishes. This change would be accentuated if the p_H values were corrected for "salt error" as just explained.

Concentration of phosphate buffer solution (M)	Optimum p_H
0·5	4·45
0·2	4·8
0·1	5·0
0·025	5·25

It has not yet been decided whether Michaelis' equations [1911] for amphoteric electrolytes are applicable in the case of living yeast to the rate of fermentation and the p_H value as applied by Michaelis and Davidsohn [1911] and Davidsohn [1913] to enzyme action, but it is interesting to note that in Table V, in which K is calculated from these equations for values of fermentation rate and p_H taken from the curve in Fig. 4 for 0·5 M phosphate, the values of K obtained on each side of the maximum are approximately constant.

Table V. 0·5 *M phosphate buffer solutions.*

p_H	Rate of fermentation cc. per 5 mins.	Relative rate of fermentation	K_a for $\frac{[H^+]}{[H^+]+K_a}$ = rel. rate	K_b for $\frac{[OH']}{[OH']+K_b}$ = rel. rate
*2·6	6·8	0·59	—	$0{\cdot}28 \times 10^{-11}$
2·65	7·0	0·60	—	$0{\cdot}30 \times 10^{-11}$
2·8	8·0	0·70	—	$0{\cdot}27 \times 10^{-11}$
2·95	9·0	0·78	—	$0{\cdot}25 \times 10^{-11}$
3·15	10·0	0·86	—	$0{\cdot}24 \times 10^{-11}$
*4·0	11·6	1·00	—	—
Opt.				
4·45	11·6	1·00	—	—
*4·9	11·6	1·00	—	—
5·9	10·0	0·86	$0{\cdot}21 \times 10^{-6}$	—
*6·1	9·3	0·80	$0{\cdot}20 \times 10^{-6}$	—
6·35	8·0	0·70	$0{\cdot}19 \times 10^{-6}$	—
6·5	7·0	0·60	$0{\cdot}21 \times 10^{-6}$	—
6·65	6·0	0·52	$0{\cdot}20 \times 10^{-6}$	—
6·85	5·0	0·43	$0{\cdot}19 \times 10^{-6}$	—
*6·95	4·4	0·38	$0{\cdot}20 \times 10^{-6}$	—

* Represents the point of observation.

No simple relations have been found to exist between the rate of fermentation and the concentration of free H_3PO_4, or of NaH_2PO_4, or of Na_2HPO_4.

When the rates of fermentation with the hydroxy-acids are compared with those obtained with phosphate buffer solutions, a very close resemblance is observed to exist between them; *e.g.* between the curve with lactic acid and that of 0·2 M phosphate; between those of α-hydroxy-*n*-butyric acid and 0·1 M phosphate; between those of β-hydroxy-*n*-butyric acid and 0·025 M and between those of hydroxy-*iso*-butyric acid and 0·025 M phosphate. From this resemblance it may be concluded that hydroxy-acids with the exception of glycollic acid do not exert any marked specific effect upon the rate of fermentation as compared with phosphate buffer solutions.

SODIUM BICARBONATE.

Table VI shows the results obtained with various concentrations of sodium bicarbonate buffer solutions and with the corresponding concentrations of phosphate buffer solutions. In order to make the bicarbonate buffer solutions, NaOH solutions of the corresponding concentrations were used. For each concentration of $NaHCO_3$, only one p_H value is possible, since, under the conditions of the experiment, the solutions are saturated with CO_2 at 25°. For the phosphate buffers Na_2HPO_4 solutions were employed. Having added the requisite amount of sugar solution, they were saturated with CO_2, yeast was added and the fermentation was carried out.

Table VI.

Sodium bicarbonate buffer solution.

Exp. 38

Concentration of bicarbonate (*M*)	Corrected rate of fermentation cc. per 5 mins.	p_H (corrected)	Relative rate of fermentation	K_c for $\frac{[H^+]}{[H^+]+K_c}$ = rel. rate
0·5	4·5	7·3	0·417	$0·70 \times 10^{-7}$
0·2	7·3	7·0	0·676	$0·48 \times 10^{-7}$
0·1	8·9	6·8	0·824	$0·34 \times 10^{-7}$
0·05	9·7	6·5	0·898	$0·36 \times 10^{-7}$
0·025	10·8	6·2	1·000	—

Sodium phosphate buffer solution.

Exp. 39

Concentration of phosphate (*M*)	Corrected rate of fermentation cc. per 5 mins.	p_H (corrected)	Relative rate of fermentation	K_c for $\frac{[H^+]}{[H^+]+K_c}$ = rel. rate
0·5	4·4	6·7	0·373	$3·36 \times 10^{-7}$
0·2	7·6	6·6	0·644	$1·38 \times 10^{-7}$
0·1	9·7	6·5	0·822	$0·69 \times 10^{-7}$
0·05	10·8	6·3	0·915	$0·41 \times 10^{-7}$
0·025	11·8	6·1	1·000	—

The p_H values in Table VI were corrected for salt error and for the barometric pressure under which the saturation of CO_2 was carried out from the values obtained electrometrically by Biilmann and Katagiri [1927] under the same experimental conditions.

In Fig. 5 (*a*) in which the rates of fermentation shown in Table VI are plotted against the p_H, it is clearly seen that the curve with bicarbonate buffer solutions is of a similar nature to that obtained with phosphate buffer solutions, but they are not perfectly identical, since in the latter curve the rate of fermentation diminishes more rapidly than in the former when the p_H value increases.

When the rates of fermentation are plotted against the concentration of total salts (Fig. 5 (*b*)), the bicarbonate and phosphate curves are again very similar, although they are not identical, but in the latter curve the rate of fermentation diminishes a little more rapidly as the concentration increases.

When Michaelis' formula for amphoteric electrolytes is applied to the

results obtained with bicarbonate and phosphate buffer solutions, the values of K_c (Table VI) vary as is to be expected in both cases with the concentration of total salt. When the values of K_c are plotted against the molar concentration of the salts (Fig. 6), a linear relation is found to exist. The inclination of the phosphate curve is greater than that of the bicarbonate curve as was to be expected from Fig. 5 (*a*) and (*b*).

Whilst the interpretation of these results is not very clear, it may be considered that with the bicarbonate buffer solution, the rate of fermentation is more exclusively controlled by the p_H value than with the phosphate buffer solution.

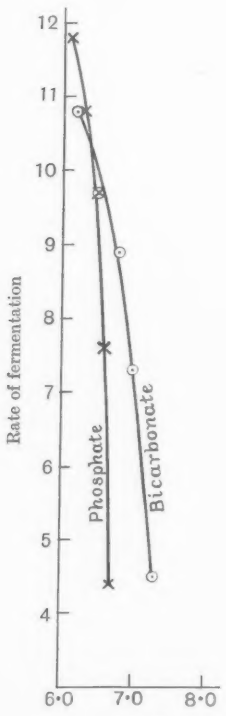

Fig. 5 (*a*)

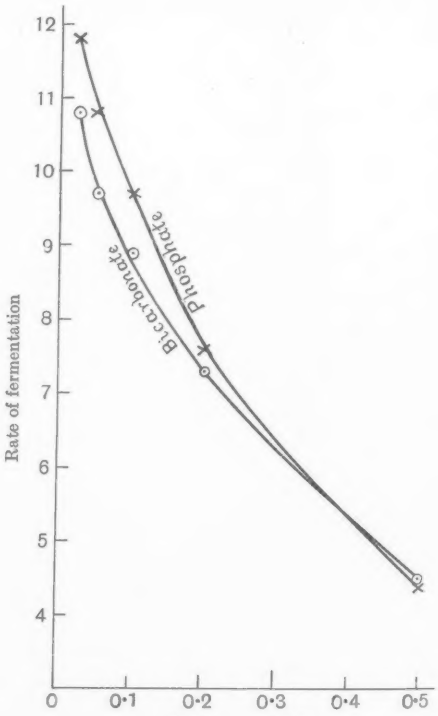

Fig. 5 (*b*)

Summary.

(1) Fermentation by living yeast was observed with various concentrations of propionate, butyrate, *iso*-butyrate, glycollate, lactate, α- and β-hydroxy-butyrate, hydroxy-*iso*-butyrate, phosphate and bicarbonate buffer solutions composed in each case of the acid and its sodium salt.

(2) The simple fatty acids have an inhibiting effect on the rate of fermentation, and it is suggested in each case that a hyperbolic relation exists between the rate of fermentation and the concentration of free acid.

(3) The specific effect of these fatty acids is nearly as great as that of acetic acid, but very much less than that of formic acid.

(4) The order of the specific effect of the fatty acids is not coincident with that of their partition coefficients between water and benzene or that of their molecular weights or dissociation constants. It is probably due, in a great measure, to the chemical nature of the acid itself.

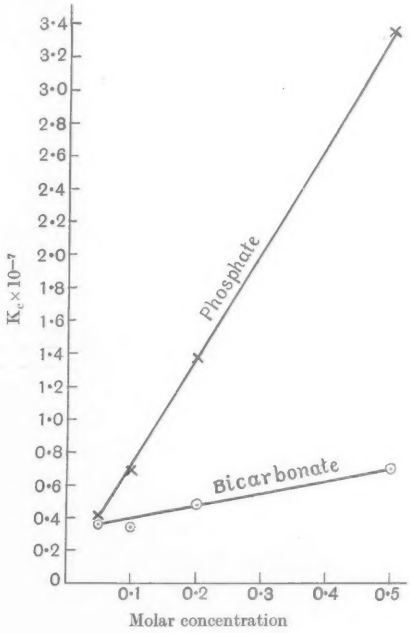

Fig. 6.

(5) No specific effect of the hydroxy-acids on the rate of fermentation as compared with phosphate buffer solutions was observed, except in the case of glycollic acid which had a slight inhibiting action similar to that of the simple fatty acids.

(6) Curves showing an optimum p_H value were obtained with phosphate buffer solutions of various concentrations. As in the case of enzyme action in general, the rates of fermentation at constant concentration of buffer solutions were observed to be principally controlled by the p_H value.

(7) Not only a different optimum p_H value but also a different sharpness of optimum p_H was observed with phosphate buffer solutions of different concentration. As the concentration diminished, the optimum p_H moved to the alkaline side.

(8) The effect of buffer solutions of sodium bicarbonate and carbonic acid is very similar to that of phosphate buffers, but the rate of fermentation is more exclusively controlled by the p_H value than is the case with the phosphate buffers.

I desire to acknowledge my great indebtedness to Prof. A. Harden for his continued interest and his valuable criticism throughout the whole of the investigation.

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LXXII. OXIDATIONS BY HYDROGEN PEROXIDE IN PRESENCE OF SULPHYDRYL COMPOUNDS.

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It has been shown recently by Thurlow [1925] and by Harrison and Thurlow [1926] that during certain biological oxidations such as that of hypoxanthine and succinic acid in presence of their respective oxidising enzymes, or sulphydryl compounds in presence of traces of iron, the secondary or induced oxidation of substances such as nitrites, lactic acid and β-hydroxybutyric acid can be brought about. These secondary oxidations were shown to occur only in presence of a suitable peroxidase, *e.g.* milk peroxidase or ferrous iron. Thurlow [1925] gave evidence that these secondary oxidations were brought about by hydrogen peroxide formed during the oxidation of the primary system by the combination of active hydrogen with molecular oxygen. In the case of the primary oxidising system hypoxanthine with xanthine oxidase, hydrogen peroxide was actually detected in the solution. The hydrogen peroxide in presence of a suitable peroxidase or activator can then bring about the secondary oxidation of other substances. During the oxidation of the sulphydryl compound, however, positive tests for the presence of hydrogen peroxide in the solution could not be obtained. This was explained by the fact that hydrogen peroxide can readily oxidise the sulphydryl group and that, consequently, hydrogen peroxide formed during the oxidation of the sulphydryl compound by oxygen would be capable of bringing about the oxidation of a further quantity of the sulphydryl compound, and hence would rapidly be used up. Similarly, hydrogen peroxide formed in other primary oxidising systems such as hypoxanthine with xanthine oxidase can bring about the oxidation of a further quantity of the oxidisable substance hypoxanthine, though in this latter case the rate of disappearance of the hydrogen peroxide is slower, requiring the presence of the xanthine oxidase. It was shown, however, by Thurlow [1925] and Harrison and Thurlow [1926] that, in spite of the fact that part of the hydrogen peroxide formed during the primary oxidation is used up in oxidising a further quantity of the oxidisable substance, nevertheless, in presence of a peroxidase another part of this hydrogen peroxide can bring about a secondary oxidation of various substances such as nitrites and lactic acid. Evidence of the same kind was given in the case of a number of oxidising systems, including those of the sulphydryl compounds cysteine and glutathione.

It was shown by Abderhalden and Wertheimer [1923] that cysteine is rapidly oxidised to cystine by hydrogen peroxide. These authors used a large excess of hydrogen peroxide in their experiments. In a recent paper, Szent-Györgyi [1926] has shown that a dilute solution of reduced glutathione is oxidised fairly rapidly by excess of hydrogen peroxide. In consequence, he considers that the presence of the sulphydryl group in the tissues will interfere with secondary oxidations brought about in presence of peroxidase by hydrogen peroxide; in other words that peroxidase cannot compete with the oxidising sulphydryl group for a share of the hydrogen peroxide to a sufficient extent to make the secondary oxidations important. This criticism does not appear to be a valid one. In the first place, the conditions in Szent-Györgyi's experiments are not physiological, since excess of hydrogen peroxide is employed and the hydrogen peroxide is all added at once. In the living cell hydrogen peroxide is formed by the primary oxidising systems themselves and is hence produced only in small quantities at a time. If it were present to a greater extent, the cell enzymes, including peroxidase itself, would be destroyed, and the widespread distribution of catalase in the tissues appears to be an additional safeguard against the accumulation of hydrogen peroxide in toxic quantities.

In the second place it is actually possible, as the following experiment will show, for a secondary oxidation by hydrogen peroxide and peroxidase to occur in presence of the sulphydryl group, and even when the hydrogen peroxide is slowly added to the system instead of being formed by the system itself. It was considered worth while to perform this experiment in order to meet Szent-Györgyi's criticism, because the mechanism involved in secondary oxidations brought about by peroxidase and hydrogen peroxide seems too important to be laid aside without due consideration. The following experiment was devised to show that even when hydrogen peroxide is added continuously to a sulphydryl system containing peroxidase together with a suitable oxidisable substance such as a nitrite, the hydrogen peroxide serves not only to oxidise the sulphydryl compound but also, activated by the peroxidase, to bring about the oxidation of the nitrite.

Broadly speaking, the principle of the experiment was slowly to add dilute solutions of cysteine, peroxidase and hydrogen peroxide to a solution of peroxidase containing a small quantity of sodium nitrite, and then to test for the disappearance of the nitrite by the Griess-Ilosvay reagent. The solutions were added in such a way that a small excess of the sulphydryl compound was present in the solution throughout the experiment. Oxidation of the cysteine by the air was entirely prevented by bubbling purified nitrogen through the solution, this also sufficing to keep the solution well stirred.

The cysteine solution containing 13·3 mg. of free cysteine per cc. (neutralised with sodium hydroxide) was quickly poured into a burette, the entry of air into the latter being prevented throughout the experiment by a stream of nitrogen. The peroxidase was prepared from milk by the method of

Thurlow [1925], and a saturated solution in phosphate buffer (p_H 7·6) was used (the solution being filtered and the necessary amount of sodium hydroxide being added to the solution to maintain the p_H at 7·6). The hydrogen peroxide solution was made up to be equivalent to the cysteine solution. The strength of the hydrogen peroxide solution was checked at the beginning of the experiment by a direct titration of the cysteine solution, a known volume of the latter being made alkaline with ammonium hydroxide, warmed, and titrated with the hydrogen peroxide, using the nitroprusside test as an external indicator.

A solution of 0·1 mg. of sodium nitrite in 20 cc. of peroxidase was contained in a thick glass boiling-tube, a bulb being blown in the upper part of the latter to increase its capacity and to reduce frothing of the protein-containing solution. The tube was fitted with a rubber stopper through which passed an entry and narrow exit tube for nitrogen and three short pieces of glass tubing each drawn out to a fine jet. Three burettes containing the cysteine, hydrogen peroxide and peroxidase respectively, were attached by short pieces of rubber tubing to the three glass tubes the jets of which had previously been adjusted to dip well under the surface of the liquid in the boiling-tube. Before putting in the rubber stopper, the burettes were turned on until all the air in the glass tubes was displaced by liquid. After drying the outside of the tubes with filter paper, the stopper was put into the boiling-tube and a stream of nitrogen (purified by passing through four vessels of pyrogallol) was bubbled through the solution. After all the air had been driven out 1·0 cc. of cysteine was added from the burette, and the tube was immersed in a water-bath at 37°. An excess of cysteine was thus present at the beginning and the excess was maintained throughout the experiment. The cysteine, peroxidase and hydrogen peroxide were now run in from the burettes in successive small quantities in the following order, namely, 0·2 cc. cysteine, 0·5 cc. peroxidase, 0·2 cc. hydrogen peroxide. (The peroxidase was added to replace that which was destroyed during the experiment.) The duration of the experiment was about one and a half hours, and in all 7·5 cc. cysteine, 20·0 cc. peroxidase and 7·5 cc. hydrogen peroxide were added. The tube was now removed from the water-bath and 4·0 cc. of water were added (by removing the nitrogen exit tube), making the total volume of the solution up to 60 cc. In the tests described below, this solution will be called solution B. A fairly rapid stream of nitrogen was maintained to prevent the entry of oxygen, and quantities of the solution were removed for testing by replacing the nitrogen exit tube by a pipette. A test portion of the solution gave a strong nitroprusside test at the end of the experiment, showing that excess of cysteine was present.

In order to test samples of the solution for the disappearance of nitrite, it was necessary first to remove the excess of cysteine, as the latter was found to inhibit the development of the colour in the Griess-Ilosvay test. This was effected by making the solution alkaline and adding hydrogen peroxide, after

destruction of the peroxidase in the solution by heat or by treatment with concentrated hydrochloric acid. (Haas and Lee [1924] have shown that nitrites are not oxidised by hydrogen peroxide in the absence of peroxidase.)

Three tests were carried out for the disappearance of nitrite as follows.

1. A control solution containing 40 cc. peroxidase and 0·1 mg. sodium nitrite was made up to 60 cc. with water. This would represent the concentration of nitrite in the experimental solution if none had been oxidised. 2·5 cc. of this control solution A and 2·5 cc. of the experimental solution B from the boiling-tube were pipetted respectively into two test tubes, each containing 1 cc. of concentrated hydrochloric acid which destroyed the peroxidase. After shaking, the solutions were made alkaline with 1 cc. of concentrated ammonium hydroxide and 0·1 cc. of the hydrogen peroxide solution was added to each to oxidise the cysteine remaining in solution B. (This hydrogen peroxide cannot oxidise any nitrite present since the peroxidase has been destroyed.) After shaking, and confirming the absence of cysteine in B, 2·0 cc. of Griess-Ilosvay reagent were added to each, and the tubes were allowed to stand a few minutes for development of colour. The solution B gave a completely negative test for nitrite, whereas the control solution A gave a definitely positive reaction for nitrite.

2. 2·5 cc. of the control solution A and of the test solution B were taken and the peroxidase in the two solutions was destroyed by boiling. After cooling, 0·1 cc. of ammonium hydroxide and 0·1 cc. of hydrogen peroxide were added. The solutions were well shaken, the coagulated protein being broken up with a glass rod, and 1 cc. of Griess-Ilosvay reagent was added to each. After standing for a few minutes, the control solution A gave a strongly positive test for nitrite, while the experimental solution B gave a completely negative reaction for nitrite.

3. Two quantities of 2·5 cc. of the solution B were pipetted from the boiling-tube and to one was added 0·05 cc. of sodium nitrite solution containing 0·00084 mg. The two solutions were then treated in the manner described in Test 2. The solution, to which 0·05 cc. of nitrite had been added before testing, gave a definitely positive test for nitrite, while the other solution, which had originally contained five times this amount of nitrite, gave a completely negative test. This last test showed that one-fifth of the quantity of nitrite which was originally present in the solution could readily be detected by this method of testing. Further, this last test gives confirmation that the disappearance of nitrite is not due to its oxidation by the hydrogen peroxide added during the test, the peroxidase having previously been destroyed.

The whole experiment was repeated under the same experimental conditions and identical results were obtained.

It is evident, therefore, that hydrogen peroxide in presence of peroxidase brings about the oxidation of nitrites in a solution containing a sulphydryl compound. No attempt was made to find the maximum quantity of nitrite

which could be oxidised by a given quantity of hydrogen peroxide and peroxidase. It was desired merely to show that such an oxidation actually could take place to a measurable extent *in vitro* in the presence of a sulphydryl compound. In our present state of knowledge it is impossible fully to imitate *in vitro* the effects on chemical reactions produced *in vivo* by the specialised structure of the living cell, and such factors as selective permeability and selective adsorption may well play an important part in determining in what proportion the hydrogen peroxide is shared between oxidising sulphydryl compounds and between bringing about, in presence of peroxidase, secondary oxidations in the living cell.

SUMMARY.

Experimental evidence is given to show that the presence of sulphydryl compounds does not prevent the oxidation of sodium nitrite by hydrogen peroxide in presence of milk peroxidase.

The bearing of this on secondary oxidations by hydrogen peroxide in the living cell is discussed.

The author wishes to express his sincere thanks to Professor E. Mellanby, F.R.S., for his interest and encouragement.

He also wishes to express his indebtedness for a Keddey Fletcher-Warr Studentship, University of London, held during the course of this work.

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LXXIII. EXPERIMENTS ON THE FORMATION OF SUCCINIC ACID IN THE BODY.

PART I. THE DETERMINATION OF SUCCINIC ACID AND ITS FORMATION IN MUSCLE AND LIVER PULP.

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(*Received April 4th, 1927.*)

In several recent publications [*e.g.* Ascher, 1925; Burn and Marks, 1926] considerable support has been given to the view that the liver normally, to some extent, converts fat into reducing sugar, and that this process becomes increasingly important when the animal is deprived of its glycogen supplies. Nothing is yet known, however, of the chemical mechanism of such conversion, nor does the theory of β-oxidation of fats readily provide an obvious mechanism.

Two suggestions have been made. It has been shown [Clutterbuck and Raper, 1926] that acetoacetic acid on oxidation with hydrogen peroxide, which, according to Dakin [1908, 1, 2, 3; 1910], simulates certain physiological oxidations, yields α-hydroxyacetoacetic acid. It was therefore suggested that in the liver acetoacetic acid, known to arise in the metabolism of fat, might give rise successively to α-hydroxyacetoacetic acid, diketobutyric acid and finally, by loss of carbon dioxide, to methylglyoxal, which is readily converted by glyoxalase [Dakin and Dudley, 1913, 1914] to lactic acid, and to glucose.

Consideration of the chemistry of muscle leads to the second suggestion. It has been known for a long time [Einbeck, 1913, 1914] that fresh muscle extracts contain considerable amounts of succinic acid, which has not arisen from putrefaction as previously suggested [Wolff, 1903], and the amount in such extracts has recently been determined [Moyle, 1924]. Muscle tissue also contains a very powerful enzyme which acts on succinic acid [Thunberg, 1909; Batelli and Stern, 1911], and is remarkably specific. The enzyme, according to Batelli and Stern, converts succinic acid into optically inactive malic acid, but their identification of the acid was not very convincing. Einbeck [1914] regarded fumaric acid and not malic acid as the sole product, but later [1919] he showed that the enzyme first converted succinic to fumaric acid and then by a balanced reaction to a mixture of fumaric (25 %) and malic (75 %) acids. Dakin [1922] showed that fumaric acid, reacting with muscle

pulp, formed exclusively *laevo*-malic and not the inactive acid, and he pointed out that this asymmetric synthesis was of additional interest from the fact of the stereochemical similarity of *l*-malic and *d*-lactic acids. The succinic acid formed *in vivo* may, therefore, represent an intermediate stage of a special type of sugar formation by way of fumaric, malic and lactic acids. It is known that malic acid yields glucose in the diabetic organism and that succinic acid gives "extra glucose" in phloridzinised animals [Ringer, Frankel and Jones, 1913].

Nothing is known as to the origin of succinic acid in the body. Although succinoxidase is so very powerful, specific and widely distributed, yet its only function appears at the present time to be that of dealing with any small amounts of succinic acid supposed to arise from glutaminic acid during the metabolism of proteins. It is interesting to enquire whether succinic acid might not arise by direct oxidation of fatty acids in the body. During oxidations *in vitro* with hydrogen peroxide, formation of succinic acid very often occurs. Thus butyric acid [Cahen and Hurtley, 1917] gives, besides acetoacetic acid, a considerable amount of succinic acid. Also caproic, oenanthic, caprylic, myristic, palmitic and stearic acids [Clutterbuck and Raper, 1925] give, besides the β-oxidation product, γ-keto-acids; and the first three, δ-keto-acids also. These on further oxidation form succinic acid. Thunberg has also suggested that acetic acid may yield succinic acid *in vivo*

$$2CH_3.COOH \rightarrow COOH.CH_2.CH_2COOH + H_2O.$$

Knoop and Gehrke [1925] oxidised acetic acid and acetone by standing at the ordinary temperature for a long time with hydrogen peroxide, and although but little oxidation of acetic acid occurred, they obtained from acetone considerable amounts of a mixture of tartaric, malic and succinic acids. It has also been shown [Clutterbuck and Raper, 1926] that acetoacetic acid gives on oxidation with hydrogen peroxide, besides α-hydroxyacetoacetic acid, a considerable amount of acetonylacetoacetic acid and a little ethyl isocarbo pyrotritartrate, and it was suggested that these had arisen from the intermediate formation of diacetylsuccinic acid. It was considered that possibly diacetylsuccinic acid might similarly arise from acetoacetic acid *in vivo*

$$2CH_3COCH_2COOH \rightarrow \begin{matrix} CH_3COCH.COOH \\ | \\ CH_3COCH.COOH \end{matrix}$$

and give rise on hydrolysis to succinic acid.

Kay and Raper [1922] injected atropic acid into dogs and isolated a small amount of succinic acid from the urine. It would seem almost impossible that this acid could have been formed from the substance injected. Atropic acid, however, on injection caused some damage to the kidney, as evidenced by haemoglobinuria, and the explanation of the appearance of succinic acid possibly lies in this fact. This question is being further investigated.

The above evidence has led the author to embark upon a study of the

formation of succinic acid with the object of throwing some light on its origin. The method of Moyle [1924] gives excellent results with very small amounts of the acid. It is, however, rather long and tedious, and is not suitable for our purpose on account of the extremely great activity of succinoxidase, which is capable of disposing of large amounts of succinic acid in a short time. It seemed better, therefore, to attempt to follow the production of this acid not directly but in terms of the *l*-malic acid arising from it by the action of the enzyme, the malic acid being not readily further attacked in experiments with the isolated tissue and being rapidly determinable by means of its optical rotation. In the present paper, a polarimetric method of this kind is described. It has been used to follow the conversion of succinic and fumaric acids to malic acid, and the effect on these reactions of the addition of cyanide has been determined. It has also been adapted to the detection of smaller amounts of succinic acid and, in order to test the above suggestions, it was applied to determine whether succinic acid was produced in liver and muscle pulp when the following substances were added: sodium acetate, acetoacetate, α-ketoglutarate, δ-ketohexoate, diacetylsuccinate and acetone. It was found that although liver and muscle, under the conditions of the experiments, were able very readily to oxidise (*i.e.* dehydrogenate) succinic acid, yet they were not able to oxidise any of the above substances in the way that hydrogen peroxide could do. Thus sodium acetate was not attacked, acetone, acetoacetic and diacetylsuccinic acids did not give rise to succinic acid, δ-ketohexoic acid, which was obtained by oxidation of hexoic acid with hydrogen peroxide and which very readily gave succinic acid on oxidising further, did not give an amount detectable by the method, and α-ketoglutaric acid, which may be expected to yield succinic acid in the intact animal, did not do so in these experiments. The *l*-malic acid, formed from succinic acid, also remains as such, although in the intact animal it is supposed to lose carbon dioxide yielding lactic acid, since it yields glucose in the diabetic animal. Similar differences between results under experimental conditions and in the intact animal are found when the liver is perfused. Raper and Smith [1926] found that only 80 % of the theoretical acetone could be recovered from perfused butyric acid, and concluded that the liver oxidised the remaining 20 % without the appearance of acetone bodies. On the other hand, Snapper and Greenbaum [1927] found that when either acetoacetic or β-hydroxybutyric acid was perfused, the latter was absorbed by the liver tissue, the amount contained by the liver being sometimes as great as six to eight times the amount expected from the acetone content of the blood. Taking this absorption into account, the amount of these acids further oxidised is very small indeed. The perfused liver, however, in that it has been shown to attack a large number of amino-acids, does appear to be much more powerfully oxidising than the isolated liver without its blood supply. It is proposed therefore to investigate the effect of perfusion of some of the substances referred to above through the surviving liver.

Experimental.

The immediate purpose of the experiments was to find out what substances, on addition to liver and muscle pulp, could result in the formation of succinic acid. Such substances, incubated with the pulp in presence of oxygen, should give rise to *l*-malic acid and be determinable, therefore, in virtue of the resulting change in rotation. In order to decide whether the formation of succinic acid could be detected in this way, a method was first devised for following the changes in rotation obtained when succinic acid is added to liver and muscle pulp.

Detection of succinic acid, added to muscle and liver pulp.

The method is an adaptation of that used by Dakin [1922] with fumaric acid, and depends on the fact that the normal small rotation of *l*-malic acid in water, $[\alpha]_D^{20°} - 1{\cdot}7°$, is increased in presence of uranium acetate to $[\alpha]_D^{20°} - 482°$. The muscle and liver used in these experiments were obtained from rabbits under sterile conditions. After killing the animal, the fur near the line of incision was removed, the animal washed with water and lysol, the skin carefully retracted and clipped back, and the back and leg muscles excised, using sterilised instruments. The dishes, mincer, container and pipettes were also sterilised. In each experiment, 5 g. of succinic acid were neutralised to litmus, diluted to 500 cc. and added to 100 g. minced sterile muscle or to 30 g. minced liver, from which the gall bladder and main portal tracts had been removed before mincing. The material was placed in a sterilised bottle (capacity about 1200 cc.) and oxygenated by passing in the gas through a cotton wool plug and sterilised tube, until the whole of the air was displaced with oxygen. The bottle was then closed with a rubber bung, wired and rotated in a thermostat at 38°. It was opened hourly for re-oxygenation and removal of samples. Each sample (25 cc.) was heat-coagulated on a boiling water-bath, 10 g. solid uranium acetate were added, made up to 100 cc., cooled, filtered and transferred to a polarimeter tube (4 decimetre) and the rotation determined. If the filtered solution was allowed to stand, occasionally a very slight opalescence appeared which could not be removed by filtration and which made it difficult to obtain a reading. Such solutions on standing longer, especially in sunlight, deposited a small amount of a flocculent precipitate and on filtration were clear. In two experiments also in which much larger amounts of a liver containing a considerable amount of glycogen were used, the glycogen opalescence made readings difficult.

The method was first applied to the incubated pulp without addition of sodium succinate. The zero reading was quite constant for different samples of muscle, and the change in rotation during 6 hours' incubation either nil or slightly dextro (+ 0·2°).

The changes in rotation in one of a number of experiments in which

5 g. succinic and 5 g. fumaric acids (as sodium salts) respectively had been added to 100 g. minced sterile muscle were as follows:

	5 g. succinic acid added			5 g. fumaric acid added		
Muscle sample	Time hours	Rotation	*l*-Malic acid formed (g.)	Time hours	Rotation	*l*-Malic acid formed (g.)
1	0	0·0 °	0·0	0	0·0 °	0·0
2	1	−0·30	0·36	1	−1·22	1·46
3	2	−0·63	0·75	2	−1·96	2·34
4	3	−0·88	1·05	3	−2·64	3·15
5	4	−1·18	1·41	4	−2·82	3·36
6	5	−1·42	1·69	5	−2·90	3·46
7	6	−1·64	1·96	6	−2·90	3·46
8	7	−1·83	2·18			
9	8	−2·01	2·40			
10	10	−2·36	2·82			
11	11	−2·40	2·86			
12	12	−2·40	2·86			
13	13	−2·40	2·86			

In calculating the *l*-malic acid formed, a small correction was applied for the volume of water contained respectively by the muscle and liver.

Replacing muscle by 30 g. liver, the following results were obtained:

	5 g. succinic acid added			5 g. fumaric acid added		
Liver sample	Time hours	Rotation	*l*-Malic acid formed (g.)	Time hours	Rotation	*l*-Malic acid formed (g.)
1	0	0·0 °	0·0	0	0·0 °	0·0
2	1	−0·59	0·64	0·5	−1·53	1·65
3	2	−1·22	1·32	1·0	−2·46	2·65
4	3	−1·90	2·05	1·25	−2·84	3·06
5	4	−2·58	2·79	1·50	−3·02	3·26
6	5	−3·12	3·37	1·75	−3·20	3·45
7	6	−3·28	3·54	2	−3·27	3·52
8	7	−3·28	3·54	3	−3·27	3·52

The experiments with 100 g. sterile muscle and 5 g. succinic and fumaric acids (as sodium salts) were then repeated with sufficient potassium cyanide added to make the solution 0·03 % KCN. The results are as follows:

	5 g. succinic acid added			5 g. fumaric acid added		
Muscle sample	Time hours	Rotation	*l*-Malic acid formed (g.)	Time hours	Rotation	*l*-Malic acid formed (g.)
1	0	0·0 °	0·0	0	0·0 °	0·0
2	2	−0·62	0·74	1	−1·22	1·46
3	3	−0·64	0·76	2	−1·94	2·31
4	4	−0·64	0·76	3	−2·58	3·08
5	6	−0·64	0·76	4	−2·79	3·32
6	7	−0·64	0·76	5	−2·84	3·38
7	—	—	—	6	−2·84	3·38

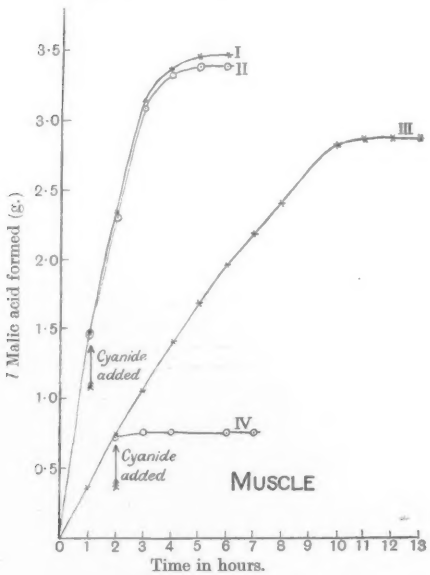
The cyanide was added, in the succinic acid experiment, after 2 hours and, in the fumaric acid experiment, after 1 hour had elapsed from the beginning of the experiment.

When these results are plotted, a number of interesting observations may be made.

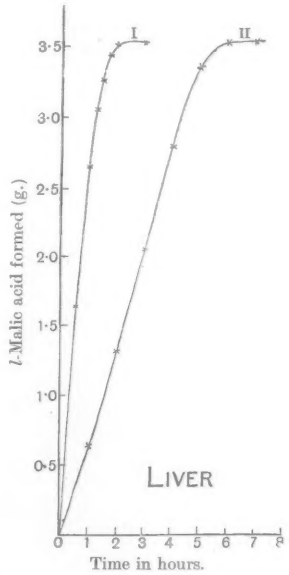
(1) Rabbit liver, weight for weight, is about eight times as active as rabbit muscle in the conversion of *both* succinic *and* fumaric acids to malic acid.

(2) The power of the liver enzyme to attack succinic and fumaric acids is remarkably great, 30 g. of liver converting 5 g. of succinic acid to the equilibrium mixture in 6 hours, and 5 g. fumaric acid in 2 hours. This will render isolation of these acids in the body an extremely difficult task. The velocity of the oxidation reaction is obviously much less than that of the subsequent addition of water and not *vice versa*, as has often been supposed.

(3) The succinic acid reaction with muscle does not attain quite to the same equilibrium as the fumaric acid reaction. This is probably due to the longer time required in the former, with resulting onset of bacterial infection, which often begins to make itself evident after 10–12 hours.



I. Fumaric acid + muscle.
II. Fumaric acid + muscle + 0·03 % KCN.
III. Succinic acid + muscle.
IV. Succinic acid + muscle + 0·03 % KCN.



I. Fumaric acid + liver.
II. Succinic acid + liver.

(4) Cyanide (0·03 %) completely stops the succinic acid reaction but has no effect on the fumaric acid reaction. The latter is therefore a simple addition of the elements of water and not an oxido-reduction, and the action of cyanide is strictly localised to the oxidation phase.

The curves above are readily reproducible with different samples of muscle and liver, the slight differences affecting the equilibrium point rather than direction of the curves.

Isolation and identification of the l-malic acid produced.

The *l*-malic acid formed by the oxidation of succinic acid with tissue pulp was isolated and identified in the following way. The resulting oxidation mixture was heated in a boiling water-bath to coagulate the proteins and filtered through muslin. The coagulated protein was washed twice in hot water and again filtered through muslin. The combined filtrate and washings were then filtered through papers and the filtrate evaporated under reduced pressure to about 200 cc., a concentrated solution of lead acetate (20 g.) added and then a little ammonia until the liquid was just alkaline, and finally about one-third the volume of alcohol, and the whole allowed to stand overnight. The precipitate containing malic and fumaric acids was filtered off, washed with a little cold water, decomposed with a slight excess of sulphuric acid and filtered. The filtrate was made alkaline with barium hydroxide and the precipitated phosphate and fumarate filtered off. The filtrate was then neutralised, the malic acid precipitated as the silver salt and recovered by passing hydrogen sulphide into its suspension in water, filtered and evaporated. The crude acid was then dissolved in a little water and extracted with butyl alcohol under reduced pressure by Dakin's [1920] method. For final identification, the *l*-malic acid was converted into the cinchonine salt, M.P. 197–198°, and shown to be identical with a synthetic sample obtained by resolving inactive malic acid by means of cinchonine [Dakin, 1924]. The mixed melting point and rotations were identical with those obtained by Dakin.

Limits of the method. In the preceding experiments, a 1 % solution of succinic and fumaric acids had been used. It was next attempted to find out if the presence of much smaller amounts of succinic acid could be detected by this rotational method. In this series of experiments, 100 g. muscle or 30 g. liver were added to 500 cc. of a neutralised 0·1 % solution of succinic and fumaric acids, and, for heat-coagulation, a sample of 100 cc. was removed, heat-coagulated, uranium acetate added and the rotation found as before. The following are typical results with muscle:

Acid	Time (hours)	Change in rotation	*l*-Malic acid formed (g.)
Succinic	6	−0·90°	0·27
Fumaric	6	−1·12	0·33

These results obtained with 0·1 % solutions of the acids compare very well with the equilibrium values obtained previously with 1 % solutions, for succinic acid 2·86, and for fumaric acid, 3·46. The above figures show that although the method has only been used for detection of amounts of succinic acid of this order, yet it is capable of greater delicacy by taking larger samples or by using smaller changes in rotation.

Application of the method. The method, in the adapted form, was then used in attempts to detect the formation of succinic acid in liver and muscle pulp from the following possible precursors, viz. acetone, sodium acetate, α-ketoglutarate, acetoacetate, diacetylsuccinate, and δ-ketohexoate. The sub-

stances in 500 cc. of water were added to the muscle or liver pulp (in place of the succinate) and rotated in oxygen for 6 hours, reoxygenating hourly. The difference in optical rotation at the beginning and end of the period was in every case slightly dextro (as in a blank determination). It would appear, therefore, that although the above substances (except diacetylsuccinate) have been shown to yield succinic acid with hydrogen peroxide, they are either not similarly oxidised by muscle and liver pulp under the conditions of these experiments, or the amount of succinic acid formed is so small that it cannot be detected by the method.

Preparation of δ-ketohexoic acid. [See Clutterbuck and Raper, 1925.]

Preparation of sodium diacetylsuccinate. Ethyl diacetylsuccinate was first obtained by the action of iodine on ethyl sodioacetoacetate in ether solution by the method of Rugheimer [1874], and converted into ethyl isocarbopyrotritartrate by the method of Knorr and Haber [1894], 10 g. of ethyl diacetylsuccinate yielding only 3·85 g. of the recrystallised product. This substance was converted into sodium diacetylsuccinate just before use.

Ethyl isocarbopyrotritartrate (6·8 g.) was warmed with 54·4 g. of a 20 % solution of sodium hydroxide until the solution became clear, and boiled for 6½ minutes. It was cooled under the tap and in iced water, and then added to 13·6 cc. of sulphuric acid in 102 cc. of water. The free isocarbopyrotritartaric acid was filtered off, washed, dried and dissolved in the theoretical amount of sodium hydroxide by heating for 1 minute. The sodium diacetylsuccinate thus formed was made up to 500 cc. and added to the liver pulp as before.

Preparation of α-ketoglutaric acid. By condensation of ethyl oxalate and succinate in presence of sodium ethoxide, an ester is obtained [Wislicenus, 1889], which according to Blaise and Gault [1908], on boiling with hydrochloric acid and evaporating, yields α-ketoglutaric acid. On attempting to obtain the acid by this method, b aporating on the water-bath, a viscous syrup was obtained which, as Gabriel [1909] showed, readily reacts with hydrazine and must contain a considerable amount of α-ketoglutaric acid; yet it was difficult to obtain a pure specimen by recrystallisation. The method of obtaining the pure acid was as follows. Ethyl oxalylsuccinate was first prepared by the method of Wislicenus and Waldmüller [1911]. Potassium (13·4 g.) was added to a mixture of absolute, dry ether (150 g.) and absolute alcohol (40 g.) distilled over sodium and the whole warmed under a reflux until dissolved and then cooled. Pure oxalic ester (50 g.) was then added and after 10 minutes pure succinic ester (59·5 g.) poured in with shaking. The potassium salt of the double ester began to separate immediately and after standing a few hours the solution had set to a solid mass. The ether-alcohol and any unchanged ester were squeezed out of the solid mass by means of a press, the solid again shaken with ether and the ether similarly removed.

The potassium salt was then suspended in water, acidified and the ester

extracted with ether, the ethereal solution dried over sodium sulphate, and the ether removed. The ester was then boiled for 2 hours under reflux with six times its weight of diluted hydrochloric acid (1 in 2) and evaporated *in vacuo* (1 mm.) from a water-bath completely to dryness, when almost pure α-ketoglutaric acid set solid in the distilling flask. If the evaporation is not carried out *in vacuo*, other products, probably of condensation, arise and cause the difficulty in obtaining a pure product by recrystallisation. The product was dissolved in a little warm acetic acid and allowed to stand. After several hours, a little acid had separated and this was filtered off and the solution stirred and allowed to stand overnight. The bulk of the acid now separated and this second crop was practically pure and melted at 110–112°. It was once more recrystallised from acetic acid and melted at 112°. It readily gave a semicarbazone, M.P. 220°.

Summary.

(1) A polarimetric method for following the conversion of succinic acid to fumaric and malic acids is described.

(2) Using the minced organs, rabbit liver is eight times as active, weight for weight, as rabbit muscle in bringing about the conversion *both* of succinic to fumaric acid and of fumaric to malic acid.

(3) Using the method, with the minced organs, no production of succinic acid from the following possible precursors could be detected: acetone, sodium acetate, acetoacetate, α-ketoglutarate, δ-ketohexoate and diacetylsuccinate.

(4) Cyanide, while inhibiting completely the change from succinic to fumaric acid, has no action on the conversion of fumaric to malic acid. The latter change is therefore not an oxido-reduction process but a simple addition of the elements of water.

In conclusion, the author desires to thank Professor H. S. Raper for helpful suggestions and criticism, and Mr O. N. Jones for considerable assistance in the experimental part of this work.

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LXXIV. STUDIES OF THE UREA CONTENT OF CAPILLARY AND VENOUS BLOOD.

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The question as to whether equilibrium exists between the concentrations of urea in the blood and those of the various organs and secretions has been studied by Gad-Andresen [1921]. He arrived at the conclusion that equilibrium exists as far as the secretions are concerned, with the exception of tears and sweat, where the urea content increases with increased secretion. As for the organs, Gad-Andresen's investigations are not conclusive. In muscle he finds a lower urea content than in blood, whereas the ammonia content is higher in such a proportion that the sums of the two substances are identical for muscle and blood. In the liver the concentration of urea is found to be the same as in the blood, while in fatty tissue it is less. For his determinations Gad-Andresen used a micro-method devised by himself.

Tschudi-Madsen [1924], using Van Slyke's urease method for his studies on the distribution of urea, found in all cases higher concentrations of urea in the organs than in the blood. The values for urea in exudates, transudates and cerebrospinal fluid were found to lie very close to those for serum, though slightly lower on an average.

Neither of these authors gives any data with regard to the distribution of urea in arterial and venous blood. This question has been studied by some other investigators, from whose works the following data may be quoted [from Bollman, Mann and Magath, 1924]: Poisseule and Gobley found in some cases a higher, in some cases a lower urea content in venous than in arterial blood; Picard found in some cases equal values, in some cases higher values for venous blood; Grehaut and Quincaud found equal values as far as the blood of the extremities was concerned, whilst venous blood derived from the liver and spleen contained more urea than arterial blood from the same organs. Kauffmann did not find any difference.

I. Technique.

For my determinations I first tried the micro-method devised by Göthgen [1925]. However, as the results showed too great variations, I resorted to that of Rehberg [1925, 2]. My technique was as follows: 0·5 cc. of distilled water is placed in a test-tube and 0·1 cc. blood, measured in a Hagedorn

pipette, is added. The pipette is washed through three times with the distilled water. Then 0·1 cc. of a fresh 10 % urease solution (Squibb), adjusted to p_H 7 with a phosphate buffer solution, is added. The tube, being closed with a rubber plug, is placed on the water-bath at 40° for 30 mins. Then three drops of octyl alcohol and 1 cc. saturated K_2CO_3 solution are added. Previously, however, 2 cc. $N/600$ H_2SO_4 solution, freshly prepared from $N/10$ solution, should be measured into each titration tube, one drop of octyl alcohol being added. The test-tubes and titration tubes are then connected and aeration takes place for 2·5 hours at a speed moderate enough to avoid spraying the mixture over the walls of the tubes, which gives rise to inaccuracy. One or two blank analyses are set up, and a control with a known urea solution. For the control a 60 mg. % urea solution was used.

When aeration is completed the solution is titrated with $N/30$ NaOH by means of Rehberg's microburette [1925, 1]. Methylene red as indicator was used in a 0·05 ‰ solution. The mean error in my tests was ± 1 mg. urea.

The blood samples were drawn in the morning, while the persons were still in bed and fasting; only in a few cases had they been out of bed for about an hour. The capillary blood was drawn from the ear after it had been rendered slightly hyperaemic by rubbing. Three or four samples were taken in immediate succession with a Hagedorn pipette, which was rinsed in a 3 % citrate solution before each drawing. The venous blood was taken from an arm vein, either without stasis or after a slight stasis had been procured, after I had ascertained that this made no difference to the results. The blood was evacuated into a dry test-tube, from which a number of tests, corresponding to those of capillary blood, were immediately measured off. Addition of anticoagulants proved unnecessary. The tubes were then placed in the ice-box until analysis could be undertaken a few hours later. It was experimentally substantiated that standing did not influence the constancy of the results provided the test-tubes were kept directly surrounded by ice.

II. Normal Individuals.

(*a*) *Fasting values.* I have performed analyses with blood from 18 normal persons, altogether 32 sets of analyses with capillary and venous blood. The majority of the persons tested were convalescents after some mild affection not involving metabolism.

In 27 of these 32 cases the urea concentration was found to be higher in capillary than in venous blood, with an average of 10 %. The percentage difference varied, somewhat, from time to time in the same individual as well as from one individual to another. Differences of 4 to 22 % were found. The absolute difference was on an average 2–3 mg., the lowest value being 0·9 mg. and the highest 5·7 mg.

Only in five cases out of the 32 did the venous blood show a higher urea concentration than the capillary blood, the difference being here 1 to 2 % except in a single case, where it was 20·4 % (Nos. 4 *c*, 5 *c*, 9, 12 *c* and 15 *b*,

Table I). The examination was repeated several times in four of the subjects showing the latter relation, and it appeared that on other occasions these subjects followed the main rule: higher concentration in capillary than in venous blood. The analysis which presented a much higher concentration in venous blood (20·4 %) was one in a set performed during 6 hours of fasting with the purpose of determining the urea level during this period. It appeared that there were only slight variations of the urea concentration. At the beginning and termination of the experiment the venous blood was also examined. The initial sample of venous blood had, as stated, a higher urea concentration than the capillary blood, whilst the final sample of venous blood showed a lower concentration of urea than the capillary blood drawn simultaneously (14 %).

Table I. *Normal individuals.*

No.	Sex	Age	Urea content, mg. per 100 cc.		Difference	
			Capillary blood	Venous blood	mg.	Per cent. in relation to lowest value
1	♂	12	31·6	27·6	4·0	14·4
2	,,	22	35·5	32·7	2·8	8·5
3 *a*	♀	23	27·8	25·0	2·8	11·2
3 *b*	,,	,,	32·8	29·8	3·0	10·0
4 *a*	♂	24	20·1	19·2	0·9	4·6
4 *b*	,,	,,	24·0	21·0	3·0	14·2
4 *c*	,,	,,	22·7	23·3	0·6	2·6
5 *a*	♀	26	16·8	14·3	2·5	17·4
5 *b*	,,	,,	17·8	16·8	1·0	5·9
5 *c*	,,	,,	19·4	19·7	0·3	1·5
6	♀	27	36·1	33·7	2·4	7·1
7	,,	29	26·7	24·1	2·6	10·7
8	♂	,,	27·5	24·2	3·3	13·6
9	,,	,,	28·5	29·0	0·5	1·7
10	♀	30	37·9	33·5	4·4	13·1
11	♂	31	40·5	37·3	3·2	8·5
12 *a*	,,	34	31·5	30·0	1·5	5·0
12 *b*	,,	,,	31·5	30·1	1·4	4·6
12 *c*	,,	,,	23·0	27·7	4·7	20·4
13 *a*	,,	35	22·8	18·6	4·2	22·5
13 *b*	,,	,,	40·7	35·0	5·7	16·2
14	,,	36	29·5	25·4	4·1	16·1
15 *a*	,,	40	38·9	36·5	2·4	6·5
15 *b*	,,	,,	39·1	39·9	0·8	2·0
15 *c*	,,	,,	42·1	38·9	3·2	8·2
15 *d*	,,	,,	43·8	41·4	2·4	5·7
15 *e*	,,	,,	47·2	44·3	2·9	6·5
15 *f*	,,	,,	48·8	45·5	3·3	7·2
15 *g*	,,	,,	54·3	50·2	4·1	8·1
16	,,	42	36·9	33·3	3·6	10·8
17	,,	,,	42·0	38·0	4·0	10·5
18	,,	45	46·2	42·3	3·9	9·2

(*b*) *Results after ingestion of various substances.* In two normal persons I undertook a series of analyses after the ingestion of various substances. In these cases the blood samples were taken from the ear at an hour's interval, venous blood being drawn two or three times during the course of the experiment.

In one person the substances ingested were (1) urea, 15 g., (2) protein, 35 g., (3) a diet poor in protein, consisting of French beans, butter, coffee with cream, and some rusks.

The fasting blood-urea in this person ranged on an average somewhat higher than the usual value for normals; he was however apparently a perfectly healthy man with normal urine and normal blood-pressure.

With another person analyses were undertaken after the ingestion of 70 g. fat + 70 g. starch, and after 70 g. glucose.

Following the ingestion of 15 g. urea the blood-urea showed a rise from 48 to 74 mg. in the course of 2 hours. After 10 hours the curve had not fallen to the initial level. In these cases the urea content of the capillary blood was 5–7 % higher than that of the venous blood.

Table II. 15 *g. urea test.*

♂ 40 years. (No. 15.)

	Urea content, mg. per 100 cc.		Difference	
	Capillary blood	Venous blood	mg.	Per cent. in relation to lowest value
Before intake	48·8	45·5	3·3	7·2
1 hr. after intake	69·1	—	—	—
2 hrs. „	74·5	—	—	—
3 „ „	69·4	—	—	—
4 „ „	72·1	67·0	5·1	7·6
5 „ „	68·8	—	—	—
6 „ „	68·6	—	—	—
7 „ „	62·8	59·7	3·1	5·1
8 „ „	59·3	—	—	—
9 „ „	57·4	—	—	—
10 „ „	60·6	—	—	—

After ingestion of 35 g. protein the urea concentration rose in the course of 5 hours from 39 to 46 mg., falling again to the initial value in the course of 10 hours. The urea content of the capillary blood was from 1 to 6 % higher than that of the venous blood (Table III).

Table III. 35 *g. protein test.*

Normal individual. ♂ 40 years. (No. 15.)

	Urea content, mg. per 100 cc.		Difference	
	Capillary blood	Venous blood	mg.	Per cent. in relation to lowest value
Before intake	38·9	36·5	2·4	6·5
1 hr. after intake	39·1	—	—	—
2 hrs. „	38·6	—	—	—
3 „ „	39·6	—	—	—
4 „ „	43·3	42·8	0·5	1·1
5 „ „	46·1	—	—	—
6 „ „	41·9	—	—	—
7 „ „	41·5	40·1	1·4	3·4
8 „ „	38·4	—	—	—
9 „ „	37·6	—	—	—
10 „ „	38·4	—	—	—

The results obtained after the person had been on a diet poor in protein (Table IV) showed only slight variations in the blood-urea content, just as was the case after ingestion of glucose (Table V) and fat + starch (Table VI).

The higher value for urea was found in the capillary blood except towards the close of the experiment with glucose, where the venous blood contained 3·4 % more urea than the capillary blood. The experiments thus confirmed the findings with regard to fasting values, the main rule being that the higher concentration of urea is found in capillary blood.

Table IV. *Diet poor in protein.*

	♂ 40 years. (No. 15.) Urea content, mg. per 100 cc.		Difference	
	Capillary blood	Venous blood	mg.	Per cent. in relation to lowest value
Before intake	54·3	50·2	4·1	8·1
1 hr. after intake	52·9	—	—	—
2 hrs. ,,	52·6	—	—	—
3 ,, ,,	52·4	—	—	—
4 ,, ,,	51·6	49·1	2·5	5·0
5 ,, ,,	50·3	—	—	—
6 ,, ,,	51·2	—	—	—
7 ,, ,,	50·1	49·9	0·2	0·4
8 ,, ,,	48·8	—	—	—
9 ,, ,,	51·5	—	—	—
10 ,, ,,	49·1	—	—	—

Table V. *70 g. glucose test.*

	♂ 34 years. (No. 12.) Urea content, mg. per 100 cc.		Difference	
	Capillary blood	Venous blood	mg.	Per cent. in relation to lowest value
Before intake	25·6	25·5	0·1	—
1 hr. after intake	30·1	—	—	—
2 hrs. ,,	28·8	—	—	—
3 ,, ,,	31·1	—	—	—
4 ,, ,,	30·5	—	—	—
5 ,, ,,	30·7	—	—	—
6 ,, ,,	29·1	30·1	1·0	3·4

Table VI. *70 g. fat + 70 g. starch test.*

	♂ 34 years. (No. 12.) Urea content, mg. per 100 cc.		Difference	
	Capillary blood	Venous blood	mg.	Per cent. in relation to lowest value
Before intake	31·5	30·0	1·5	5·0
1 hr. after intake	28·9	—	—	—
2 hrs. ,,	28·7	—	—	—
4 ,, ,,	29·5	—	—	—
5 ,, ,,	29·7	—	—	—
6 ,, ,,	28·5	26·3	2·2	8·3

III. Pathological Cases.

(*a*) *Fasting values.* I examined three groups of patients in whom one might expect to have found deviations from the normal with regard to the blood-urea relations. They were: 8 cases of icterus, 5 cases of various forms of nephritis and 9 diabetics.

Seven out of the eight analyses of icterus cases showed from 2 to 23 % more urea in capillary than in venous blood and only in one case was the value slightly higher for the venous blood. (No. 22, Table VII, 0·8 %.)

Table VII. *Icterus.*

No.	Sex	Age	Urea content, mg. per 100 cc. Capillary blood	Urea content, mg. per 100 cc. Venous blood	Difference mg.	Difference Per cent. in relation to lowest value	Diagnosis
19 *a*	♂	19	25·2	23·9	1·3	5·4	Icterus catarrhalis
19 *b*	,,	,,	29·0	24·7	4·3	17·4	,,
19 *c*	,,	,,	33·6	30·1	3·5	11·6	,,
20 *a*	,,	20	25·1	23·6	1·5	6·3	,,
20 *b*	,,	,,	27·1	26·5	0·6	2·2	,,
21	♀	43	12·3	10·0	2·3	23·0	,,
22	♂	63	33·9	34·2	0·3	0·8	Cholelithiasis
23	,,	66	25·9	23·8	2·1	8·8	,,

The five cases of nephritis, some of which had a considerably increased concentration of blood-urea, all showed higher values for the capillary blood.

Table VIII. *Renal diseases.*

No.	Sex	Age	Urea content mg. per 100 cc. Capillary blood	Urea content mg. per 100 cc. Venous blood	Difference mg.	Difference Per cent. in relation to lowest value	Diagnosis
24	♂	8	20·2	19·2	1·0	5·2	Glomerulonephritis
25	,,	,,	77·8	74·1	3·7	4·9	,,
26	,,	12	55·9	49·4	6·5	13·1	,,
27	,,	22	52·3	52·2	0·1	0·2	Nephrosis
28	♀	56	103·0	101·9	1·1	1·0	Nephrosclerosis

Eight of nine diabetics followed the main rule; only in one case the reverse relation was found. (No. 30, Table IX.)

Table IX. *Diabetics.*

No.	Sex	Age	Urea content, mg. per 100 cc. Capillary blood	Urea content, mg. per 100 cc. Venous blood	Difference mg.	Difference Per cent. in relation to lowest value
29	♀	24	32·2	31·5	0·7	2·2
30	♀♂	39	35·7	36·3	0·6	1·6
31	,,	48	44·2	42·0	2·2	5·2
32	,,	49	34·9	31·1	3·8	12·2
33	,,	,,	23·7	22·6	1·1	4·8
34	♀♂	55	25·4	24·6	0·8	3·2
35	♀♂	58	26·3	21·2	5·1	24·0
36	♀♂	60	87·3	83·2	4·1	4·9
37	♂	66	24·7	23·1	1·6	6·9

(*b*) *Results after ingestion of various substances.* One of the icterus cases was examined after the intake of 35 g. protein and 15 g. urea. The curves resembled very much those found for normals: an abrupt rise following the ingestion of urea (Table XI) and a slow rise after the ingestion of protein with maximum after 5 hours (Table X). In these cases also the main rule was followed with one single exception (Table X, 3 hours after ingestion).

Table X. 35 *g. protein test.*

	Icterus, ♂ 19 years. (No. 19.) Urea content, mg. per 100 cc.		Difference	
	Capillary blood	Venous blood	mg.	Per cent. in relation to lowest value
Before intake	25·2	23·9	1·3	5·4
1 hr. after intake	28·9	—	—	—
2 hrs. ,,	29·7	—	—	—
3 ,, ,,	28·1	28·4	0·3	1·0
5 ,, ,,	32·8	—	—	—
6 ,, ,,	29·0	27·1	1·9	7·0
8 ,, ,,	27·1	—	—	—

Table XI. 15 *g. urea test.*

	Icterus, ♂ 19 years. (No. 19.) Urea content, mg. per 100 cc.		Difference	
	Capillary blood	Venous blood	mg.	Per cent. in relation to lowest value
Before intake	29·0	24·7	4·3	17·4
1 hr. after intake	66·2	—	—	—
2 hrs. ,,	55·7	—	—	—
4 ,, ,,	49·2	47·6	1·6	3·3
6 ,, ,,	43·4	—	—	—
8 ,, ,,	52·0	—	—	—
10 ,, ,,	45·9	43·2	2·7	6·2

Summary.

Twenty-seven out of 32 sets of analyses of blood from 18 fasting normal individuals showed a higher concentration of urea in capillary blood (from ear-lobe) than in venous blood (from vena mediana cubiti), 10 % higher on an average. The percentage difference varied from individual to individual and also in the same individual from one day to another. Percentage differences of from 4 to 22 were found. The absolute difference was 3 mg. on an average.

In four cases the values were slightly higher for venous blood, only in a single case as much as 20 % higher.

Following the ingestion of protein, urea, diet poor in protein, fat + starch, and glucose, the main rule was again a higher concentration of urea in capillary than in venous blood in the course of the day.

Examinations of patients with diabetes, nephritis and icterus showed similar relations to those observed in normals. Only in 2 out of 22 cases was the urea content higher in venous blood.

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LXXV. THE INFLUENCE OF HEXOSEDIPHOSPHORIC ACID AND HEXOSEMONOPHOSPHORIC ACID UPON INSULIN HYPOGLYCAEMIA.

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During the last twelve years a considerable amount of evidence has accumulated which points to a carbohydrate-phosphorus complex as being an essential intermediate in the normal carbohydrate metabolism. The work of Embden and his co-workers has made it appear probable that a hexosediphosphoric acid, similar to that isolated by Harden and Young [1906] from the products of yeast fermentation, is formed as an intermediate stage in the conversion of glycogen or carbohydrate to lactic acid in the muscle.

A reduction in the concentration of the inorganic phosphate in the blood following the administration of insulin has been observed by many workers, while the exact relationship between the concentrations of the inorganic phosphate and sugar in the blood has been investigated by Eadie, Macleod and Noble [1925] and by Chaikoff, Macleod, Markowitz and Simpson [1925], who found that the inorganic phosphate in the blood showed an almost parallel decrease with the blood-sugar. If, as the evidence suggests, a carbohydrate-phosphorus compound is formed as an intermediate stage in carbohydrate metabolism, the administration of this compound might result in the complete or partial amelioration of hypoglycaemic symptoms. We have therefore tried the effect of subcutaneous injections, under such conditions, of two naturally occurring phosphoric esters of this type, hexosediphosphoric ester, and hexosemonophosphoric ester.

Robison [1922] isolated from the products of yeast fermentations a hexosemonophosphoric acid which he believed was probably a mixture of glucose- and fructose-monophosphoric acids. This conclusion has recently been supported by Meyerhof [1926], who, by determining the reducing power of the substance by Willstätter and Schudel's [1918] hypoiodite method, was able to distinguish between the aldose and ketose types of acid, since this method only estimates the reducing power of the aldose type. Meyerhof concluded

that Robison's acid was a mixture of about 60 % glucosemonophosphoric acid and 40 % of fructosemonophosphoric acid. Application of this method of analysis to hexosediphosphoric acid supports the conclusions of Young [1909] and Neuberg and Kretschmer [1911] that it consists almost entirely of fructose-diphosphoric acid.

Preparation of the acids.

The hexosemonophosphoric acid used was prepared from fructose by fermentation with a preparation of dried yeast. The general conditions of separation were similar to those used by Robison in his original investigation. The final barium salt, after drying over phosphorus pentoxide at 80° and 20 mm., possessed the following constants:

$[\alpha]^{18°}_{\text{Hg green}}$ + 16·7°. Phosphorus 7·45 %.

The barium salt was converted into sodium salt by shaking with the exact amount of a solution of sodium sulphate. The barium sulphate formed was removed by the centrifuge and the clear solution of sodium hexosemono-phosphate was concentrated by evaporation in a vacuum desiccator.

The hexosediphosphoric acid was also prepared from fructose by fermentation with dried yeast. The final product was converted into sodium salt as described above for the monophosphate. The solution of the sodium salt was free from inorganic phosphate.

Injection experiments.

The ability of the diphosphate to relieve the symptoms of insulin hypoglycaemia was first tested on rabbits, with entirely negative results. Rabbits in insulin convulsions injected with the diphosphate showed no greater recovery than the untreated animals. Certainly there was no such dramatic recovery as is observed after the injection of a corresponding amount (1 g.) of glucose.

On account of the large quantity of material required to obtain a convincing result when using rabbits, it was decided to employ mice for further tests, since they exhibit unmistakable recovery after an injection of 100 mg. of glucose.

The mice, after receiving 0·025 unit of insulin subcutaneously, were kept in special boxes heated to 37°, since under these conditions the insulin intoxication follows a well-defined course with fatal termination, unless relieved by an injection of glucose or its equivalent. In about half an hour the mice began to show typical symptoms of insulin hypoglycaemia, namely, a condition of collapse, with legs sprawling and tails erect, or actual convulsions. Five of the affected mice received a subcutaneous injection of 0·1 g. glucose; four, an injection of an equivalent amount of hexosediphosphate; five, an injection of an equivalent amount of hexosemonophosphate; and six, the same volume of saline (0·4 cc.).

All the mice which received glucose showed definite recovery in one to two minutes and survived until the end of the experiment. Although in some cases the animals were not normal until 10 or 15 minutes later, they regained the use of their legs almost immediately.

On the other hand, those which received saline showed no amelioration of symptoms. Some of them seemed to remain alive longer than would be expected in the absence of any injection, but all were dead within $\frac{1}{2}$ to 1 hour after the injection of saline.

The effect of both hexosediphosphate and hexosemonophosphate was in all respects similar to that of saline. One of the mice, which was the last to be injected with the diphosphate, was killed in a collapsed condition at the end of the experiment. All the others were already dead.

These experiments, therefore, afford no evidence that these two compounds can replace glucose in the relief of insulin hypoglycaemia, or that they are readily convertible into that substance in the animal body.

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LXXVI. HAEMOCYANIN.

PART IV. THE DEPENDENCE OF THE SHAPE OF THE OXYGEN DISSOCIATION CURVE ON THE STATE OF IONISATION OF THE PROTEIN.

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(*Received April 16th, 1927.*)

In the studies on the influence of p_H on the oxygen dissociation curves of the haemocyanins from *Homarus* and *Cancer* which were described in Parts II and III [Stedman and Stedman, 1926] of this investigation the shape of the dissociation curve was found to depend upon the relative amounts of pigment present as salt and in the uncombined form. The precise nature of this dependence was, however, obscure. This was largely due to the uncertainty which existed respecting the values of the isoelectric points of the pigments. The values determined by the method of optimum precipitation did not appear to correspond with the above relationship. Before further progress could be made it therefore became necessary to determine the isoelectric points by an independent method. This has now been done; the results obtained, which are in substantial agreement with those previously recorded, are presented in this communication.

This confirmation of the values obtained by the precipitation method has led to an extension of the investigation which is described below and which, the authors believe, has brought certain aspects of the influence of p_H on the dissociation curves into complete correspondence with the state of ionisation of the protein.

Experimental methods.

The serum employed was collected, concentrated and dialysed in the manner described in Part II; determinations of the oxygen capacity were made by the technique outlined in Part III; while p_H determinations were made electrometrically under the conditions described in Part II.

Determination of isoelectric point by cataphoresis. The apparatus employed for this purpose was that described by Michaelis [1925]. The dialysed serum (20 cc.) was mixed with 25 cc. of an acetic acid-sodium acetate buffer of known p_H and, in those cases where no precipitation occurred, introduced directly into the U-tube of the apparatus. When the buffer solution caused partial precipitation of the protein, the precipitate was removed by centrifuging and the supernatant liquid employed for the experiment. The large stopcocks of

the U-tube were then closed and the upper portion of the apparatus, after being thoroughly washed with distilled water, was filled with a mixture of 25 cc. of the same buffer solution with 20 cc. of water. Connection with reversible electrodes, consisting of copper wire dipping in solutions of copper sulphate, by means of small U-tubes containing potassium chloride in agar-agar was next made in the manner described by Michaelis. The subsequent operations were also made in accordance with the details given by this author. As source of E.M.F. an ordinary lighting circuit with a carbon filament lamp in series was employed. The direction of migration of the haemocyanin could be directly observed owing to the colour of this protein. The exact p_H of the protein-buffer mixture was determined electrometrically.

Viscosity measurements. The usual type of Ostwald viscosimeter was employed. The solutions for a given series of experiments were prepared in the following manner. A definite volume (usually 10 cc.) of the dialysed serum under examination was measured with a pipette into a graduated flask, to which were then added dilute hydrochloric acid or potassium hydroxide, in amount necessary to produce the desired change in p_H, and distilled water to the mark. Changes in p_H were, in general, effected by the addition of alkali only when the serum was on the alkaline side of the isoelectric point, and by acid only when on the acid side. The series of solutions thus prepared were allowed to stand for 15–20 hours in the ice-chest, from which they were removed individually as required for viscosimetric measurement. A definite volume (usually 8 cc.) of the haemocyanin solution was introduced into the viscosimeter which was then placed in a thermostat maintained at a temperature of 23°. The time of flow was determined after about 10 minutes.

Results.

Isoelectric point of haemocyanin from Cancer.

The results of the cataphoresis experiments are shown in Table I.

Table I.

p_H of serum-buffer mixture	Direction of migration	Remarks
6·20	To anode	Movement rapid
4·94	,,	,, slow
4·81	Mainly to anode	,, very slow
4·53	To cathode	No movement to anode
4·34	,,	Movement fairly rapid
4·05	,,	,, ,,

The isoelectric point of haemocyanin from *Cancer* thus clearly corresponds with a p_H value of about 4·7. The point of optimum precipitation has again been determined by the method described in Part II with the same result, namely, maximum precipitation occurred in the buffer solution at p_H 4·8. On allowing the precipitate to settle, however, the supernatant liquid was found to have a p_H value of 4·88. The results of the two methods are thus in substantial agreement.

Isoelectric point of haemocyanin from Homarus.

The isoelectric point of haemocyanin from *Homarus* could not be determined very accurately by cataphoresis owing to the fact that it is almost completely insoluble in acetate buffers in the region of the isoelectric point. Thus complete precipitation occurred in buffers of p_H 4·4, 4·6 and 4·8. The results of the cataphoresis experiments actually carried out are shown in Table II.

Table II.

p_H of serum-buffer mixture	Direction of migration	Remarks
4·17	To cathode	Movement fairly rapid
4·37	Mainly to cathode	„ slow
5·47	To anode	„ „

The isoelectric point thus lies between the p_H values of 4·37 and 5·47. When redetermined by the method of optimum precipitation, the greatest precipitate formed in the buffer at p_H 4·8. The supernatant liquid had a p_H of 4·84. It may be concluded that the isoelectric point of haemocyanin from *Homarus* is at about p_H 4·7.

Isoelectric point of haemocyanin from the snail (Helix pomatia).

Haemocyanin from the snail differs from the pigments from *Homarus* and *Cancer* in being relatively soluble in the region of the isoelectric point. Its solubility is not sufficiently great to prevent its precipitation from dialysed serum on the addition of acid, but is large enough to render inapplicable in its simple form the method of optimum precipitation for the determination of its isoelectric point. On the other hand, this greater solubility facilitates cataphoresis experiments. Using this method, the isoelectric point of haemocyanin from snail has been found to lie at about p_H 5·3, as can be seen from Table III.

Table III.

p_H of serum-buffer mixture	Direction of migration	Remarks
5·56	To anode	Movement slow
5·39	Mainly to anode	„ very slow
5·16	To cathode	„ „

This value is confirmed by the viscosity experiments described below.

Influence of p_H *on the viscosity of solutions of haemocyanin from* Cancer.

Tables IV and V relate to experiments carried out on the alkaline and acid sides of the isoelectric point respectively.

Influence of p_H *on the viscosity of solutions of haemocyanin from* Homarus.

Measurements have only been carried out on the alkaline side of the isoelectric point. The experimental data are given in Table VI.

Table IV.

Vol. of dialysed serum (cc.)	Vol. of 0·110*N* KOH (cc.)	Water to (cc.)	p_H	Time of flow (secs.)	Relative viscosity (water = 1)
10	0	15	6·29	60·5	1·88
10	0·3	15	6·68	65·0	2·02
10	0·6	15	7·01	67·4	2·09
10	0·9	15	7·30	70·0	2·17
10	1·2	15	7·57	70·0	2·17
10	1·5	15	7·82	68·6	2·13
10	1·8	15	7·92	66·8	2·07
10	2·1	15	8·10	65·5	2·03
10	2·4	15	8·29	62·4	1·94
10	2·7	15	8·39	60·6	1·89
10	3·0	15	8·49	59·8	1·86
10	3·3	15	8·49	60·0	1·86
10	3·6	15	8·75	62·6	1·94

Time of flow for water = 32·2.

Table V.

Vol. of dialysed serum (cc.)	Vol. of 0·080*N* HCl (cc.)	Water to (cc.)	p_H	Time of flow (secs.)	Relative viscosity (water = 1)
10	0	15	4·39	48·4	1·50
10	0·3	15	4·05	48·8	1·52
10	0·6	15	4·01	49·6	1·54
10	0·9	15	3·97	51·0	1·55
10	1·2	15	4·01	52·6	1·63
10	1·5	15	4·00	54·6	1·70
10	1·8	15	3·94	57·2	1·78
10	2·1	15	3·90	60·8	1·89
10	2·4	15	3·87	65·6	2·04

Time of flow for water = 32·2.

Table VI.

Vol. of dialysed serum (cc.)	Vol. of 0·110*N* KOH (cc.)	Water to (cc.)	p_H	Time of flow (secs.)	Relative viscosity (water = 1)
10	0	15	7·59	61·2	1·90
10	0·3	15	7·95	58·7	1·82
10	0·6	15	8·22	56·7	1·76
10	0·9	15	8·42	54·8	1·70
10	1·2	15	8·69	53·6	1·66
10	1·5	15	8·84	51·6	1·60
10	1·8	15	9·04	50·6	1·57
10	2·1	15	9·26	50·2	1·56
10	2·4	15	9·58	51·4	1·60
10	2·7	15	9·88	52·4	1·63
10	3·0	15	10·07	58·2	1·81

Time of flow for water = 32·2.

Influence of p_H *on the viscosity of solutions of haemocyanin from snail* (Helix pomatia).

The difficulty experienced in dissolving precipitated haemocyanin from *Cancer* in acid commented upon in Part III was not encountered in the case of haemocyanin from snail, presumably owing to its greater solubility and possibly, also, to its greater stability in acid solution. It was thus possible

to carry out viscosity measurements on both sides of the isoelectric point with the same sample of serum. These measurements are recorded in Table VII. In the first half of the Table the p_H of the dialysed serum was changed by the addition of alkali and in the second half by the addition of acid.

Table VII.

Vol. of dialysed serum (cc.)	Vol. of 0·110*N* KOH or 0·098*N* HCl (cc.)	Water to (cc.)	p_H	Time of flow (secs.)	Relative viscosity (water = 1)
10	1·5 (alkali)	15	10·53	80·0	2·39
10	1·2	15	9·33	64·8	1·99
10	0·9	15	8·88	67·4	2·01
10	0·6	15	8·70	68·0	2·03
10	0·3	15	8·03	54·7	1·63
10	0	15	7·46	47·2	1·41
10	0·6 (acid)	15	4·76	41·8	1·25
10	0·9	15	4·27	47·8	1·43
10	1·2	15	3·98	55·4	1·65
10	1·5	15	3·55	64·3	1·92

Time of flow for water = 33·5.

Determination of the p_H *at which the affinity of haemocyanin from* Cancer *for oxygen is at a minimum.*

The variations which occur in the affinity of haemocyanin from *Cancer* for oxygen were discussed in Part III. It became necessary in connection with the viscosity experiments described above to determine as accurately as possible the p_H at which there is minimal affinity. For this purpose advantage was taken of the fact demonstrated in Part III that this particular haemocyanin is far from completely saturated with oxygen when in equilibrium with air at a temperature of 23° and at a p_H in the neighbourhood of neutrality, and a series of experiments was carried out similar to those made in Part II in another connection and illustrated in Fig. 3 of that paper. The data, which are self-explanatory, are given in Table VIII.

Table VIII.

Vol. of dialysed haemocyanin sol (cc.)	15	15	15	15	15
Vol. of 0·110*N* KOH (cc.)	0	0	0	0·3	1·0
Vol. of 0·008*N* HCl (cc.)	3·0	1·5	0	0	0
Distilled water to (cc.)	20	20	20	20	20
p_H	6·82	7·02	7·22	7·46	7·92
Oxygen content (vols. %)	1·336	1·256	1·212	1·262	1·510
Combined oxygen (vols. %)	0·747	0·667	0·623	0·673	0·921

At an oxygen pressure corresponding with that in the atmosphere (151 mm. in the experiments recorded in Table VIII) and a temperature of 23° haemocyanin from *Cancer* thus has a minimal affinity for oxygen when at a p_H of about 7·3.

It is convenient to point out here that the expression "minimal affinity" is somewhat ambiguous. It was shown in Parts II and III that certain of the oxygen dissociation curves determined at different [H·] intersected. Obviously,

when two such curves intersect, one cannot state at which acidity the affinity of the haemocyanin is the greater unless one defines the oxygen pressure at which the affinities are being compared. In the case of the pigments from *Cancer* and *Homarus* this difficulty disappears at [H$^{\cdot}$] in the neighbourhood of neutrality, as will be evident from an inspection of the curves referred to above. When in the following pages affinities at different [H$^{\cdot}$] are compared without reference to the oxygen pressure under consideration, it must be understood that the comparison is limited to the initial portions of the curves, *i.e.* if two curves intersect the comparison is limited to oxygen pressures lower than that at which the intersection occurs.

Discussion.

The experiments described above render necessary a re-examination of the dissociation curves determined in Parts II and III. Of these, the series of curves illustrated in Figs. 1 and 2 of Part III, which relate to the haemocyanin from *Cancer*, extend over the greater p_H range and will therefore be considered in rather more detail.

It was pointed out in Part III that there were indications that the haemocyanin employed for the curve at p_H 3·84, which was the most acid solution examined, had been partly converted into a form which no longer combined reversibly with oxygen. That some drastic change in, or decomposition of, the pigment takes place at or near an acidity corresponding with this p_H receives definite confirmation from the p_H-viscosity experiments recorded above in Table V. Whilst addition of acid to the dialysed serum at p_H 4·39 produced continuous increases in its viscosity, no such change occurred in the p_H. Thus, the addition of the first 0·3 cc. of acid caused the p_H to decrease to 4·05, but the further addition of 2·1 cc. scarcely produced any appreciable effect. Evidently some peculiar change occurs in the protein at a p_H of about 4·0. Changes in the external characteristics of the solution accompany this change. Thus, the initial solution (p_H 4·39) employed for the experiments recorded in Table V exhibited a marked blue opalescence (Tyndall phenomenon) which diminished progressively with increasing content of acid, giving place in the mixture at p_H 3·87 to a clear greenish-yellow solution. The intervening mixtures had a greenish appearance which was the more pronounced the greater the blue opalescence. One is forced to the conclusion that the loss in opalescence is indicative of a loss of power to combine reversibly with oxygen, a conclusion which is reinforced by the observation, made during the p_H determinations, that the opalescence disappears entirely when hydrogen is bubbled through the solutions whereas the greenish-yellow colour is not affected by this treatment. Since, further, the loss of opalescence following the addition of acid takes place slowly, it is clear that the dissociation curve at p_H 3·84 may be subject to errors arising from this cause. Such a possibility was tacitly suggested in Part III, and in view of the above results the position of the curve must be regarded as doubtful. There then remain in the immediate

neighbourhood of the isoelectric point, which has now been definitely shown to lie at about p_H 4·7 (see Table I), two curves, one on the acid side (p_H 4·19) and one on the alkaline side (p_H 5·60). The former is steeper than the latter, but in view of the fact that the p_H interval between it and the isoelectric point is the smaller, this is not inconsistent with the occurrence of a maximal affinity of the pigment for oxygen at the isoelectric point. Unfortunately it is doubtful, owing to the insolubility of the pigment in the zone surrounding the isoelectric point and to the above-mentioned change which occurs at p_H 4, whether the existence of such a maximal affinity could be directly demonstrated. Nevertheless, the probability that it does exist seems very great, although the occurrence of a maximal affinity on the acid side of, and not at, the isoelectric point would not be inconsistent with the results discussed below.

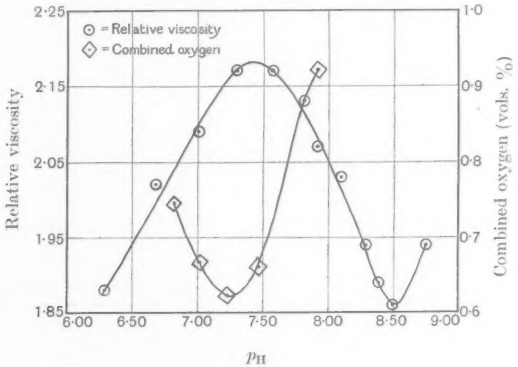

Fig. 1.

The variations in affinity which occur on the alkaline side of the isoelectric point must now be considered in connection with the viscosity determinations. For this purpose the results given in Tables IV and VIII have been plotted in Fig. 1. It can be seen that not only does the decrease in affinity which takes place as one recedes from the isoelectric point correspond, as was to be expected, with an increase in viscosity, but, what is more significant, the p_H (7·3) at which the affinity is at a minimum coincides with that at which the viscosity is at a maximum. Further, if one examines the dissociation curves illustrated in Part III it is evident that the curves (Fig. 2) determined at p_H's more acid than 7·3 are not the exact counterpart of those (Fig. 1) determined at more alkaline p_H's. On the contrary, while the latter are definitely of the familiar **S**-shape, the former do not show a marked point of inflexion. In fact, the first few points of the curves at p_H 4·19, 5·60 and 6·21 lie on straight lines passing through the origin. Whilst the possibility of a slight inflexion in the curves, such as that actually illustrated, cannot be definitely excluded it would seem that between the isoelectric point and the point of maximal

viscosity the amount of oxygen taken up in combination is, within the limits of experimental error, initially proportional to the oxygen pressure.

The viscosity experiments (Table VI) on haemocyanin from *Homarus*, the results of which are plotted in Fig. 2, are in conformity with the above relationship. Owing, however, to the more extensive zone of insolubility exhibited by this pigment in the region of the isoelectric point the authors have not hitherto succeeded in demonstrating the occurrence of either a point of maximal viscosity or one of minimal affinity. Nevertheless, it is clear from Fig. 1 in Part II that if a point of minimal affinity exist it will occur at a p_H of less than 7·76. By analogy with other proteins it is also evident from Fig. 2 that a point of maximal viscosity will likewise occur at a p_H somewhat lower than 7·59. In view of the results obtained with haemocyanin from *Cancer* there seems little doubt that these two points will coincide. One further point merits mention in connection with *Homarus*. It will be recalled that at p_H 9·65 the combination with oxygen of the pigment from this species follows closely, if

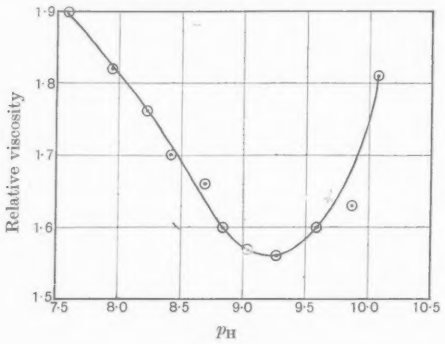

Fig. 2.

not completely, the laws applicable to homogeneous solutions. Reference to Fig. 2 shows that at this p_H the viscosity lies on the alkaline side of a minimum in the p_H-viscosity curve. The increase in viscosity which is taking place evidently represents the commencement of those processes which finally produce, in more alkaline solutions, gelation.

Although the determinations of the viscosity of solutions of haemocyanin from snail (Table VII) were made primarily with the object of confirming the position of the isoelectric point, an inspection of Fig. 3, in which the results are plotted, shows that a maximal viscosity occurs on the alkaline side of the isoelectric point at a p_H of about 8·5. It is thus possible to predict that the affinity of this haemocyanin will be at a minimum in the neighbourhood of this p_H.

The examination of further respiratory pigments will doubtless reveal relationships between viscosity and affinity similar to that demonstrated in this communication. Whilst it is to be expected that the relationship will be

of the same precise nature, it can be foreseen that it will be less marked with certain pigments. Loeb [1922] has shown that the viscosity of solutions of proteins, such as egg-albumin, which are readily crystallisable, is influenced by changes in p_H to a smaller degree than, for example, that of a gelatin solution. Whilst, as shown by Pauli and his co-workers [1925, 1926], changes of the same nature do actually occur with both types of protein, it is evident from Loeb's work that they are less pronounced in the case of the crystallisable proteins. In other words, the more readily a protein can be crystallised the less marked are its colloidal properties. The haemocyanins from *Cancer* and *Homarus* are examples of proteins with pronounced colloidal properties. As a result, the influence of p_H on both the viscosity and the power of combination with oxygen of these proteins is very great. The haemoglobins are less colloidal in nature; hence the relationship between viscosity and oxygen-combining power will not be so evident although it should be no less precise than in the case of the haemocyanins.

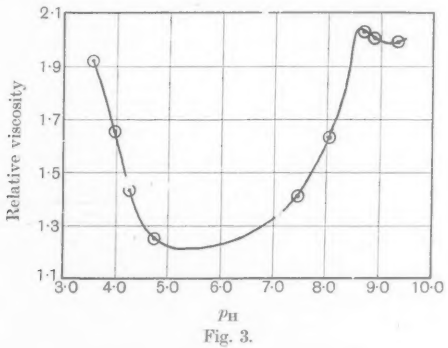

Fig. 3.

The ultimate bearing of the relationship discussed above on the mechanism of the combination between oxygen and respiratory pigments cannot yet be foreseen, but certain factors appear to be quite evident. Maximal affinity for oxygen probably corresponds with minimal ionisation and minimal viscosity, *i.e.* occurs at the isoelectric point; the alternative view that the point of maximal affinity occurs on the acid side of the isoelectric point and corresponds with maximal ionisation of the protein as cation cannot, however, be entirely excluded. As stated above, it is unfortunately scarcely possible to distinguish between these two possibilities in the case of haemocyanin from *Cancer* owing to the limits of stability of this pigment. According to Pauli [1926], maximal viscosity in proteins indicates maximal ionisation; hence, since in the case of certain haemocyanins the former corresponds with minimal affinity, it would appear that the greater the degree of ionisation (as anion) of the pigment the smaller is its affinity for oxygen. As the ionisation is repressed by increased alkalinity of the solution the affinity again increases. Finally, at $[H^{\cdot}]$ at which

the hydration processes which ultimately result in gelation have commenced the combination with oxygen takes place according to the laws for true solutions. So much seems clear from the combined experiments on *Cancer* and *Homarus* haemocyanins. Why the affinity should vary with the ionisation is not so clear, nor is it apparent why the curves on the acid and alkaline sides of the point of maximal viscosity should be of such a markedly divergent character. One factor which must certainly be taken into account in this connection is the hydration of the micellae. According to Pauli's views the point of maximal viscosity corresponds with maximal hydration of the micellae, due to the greater affinity of the ions for water, from which one might infer that the affinity for oxygen is influenced primarily by hydration and is only affected by ionisation in so far as this influences the hydration of the micellae. But the question arises: is the viscosity curve an accurate indicator of the state of hydration of the micellae? According to Smoluchowski [see Freundlich, 1926] the presence of a charge on the micellae of a sol produces an increase in the viscosity of the sol. Since maximal viscosity in a protein sol corresponds with maximal ionisation, *i.e.* with maximal charge on the micellae, it is conceivable, indeed probable, that the variation in viscosity is conditioned largely, if not mainly, by the variation in the charge on the micellae and not by variation in the state of hydration. On this basis it would be necessary to assume that the hydration of the micellae increases uniformly, or at any rate without passing through a maximum, as one recedes from the isoelectric point until finally the sol undergoes gelation. The affinity would then be directly controlled in, it must be admitted, an unknown manner by the magnitude and perhaps also the sign of the charge on the micellae, whilst the actual contour of the curve would be determined by the degree of hydration of the micellae. (The sign of the charge could only be considered of influence if it were shown that maximal affinity occurred on the acid side of the isoelectric point and corresponded with maximal ionisation of the protein as cation; such a result would be of great theoretical interest, but its consideration must be postponed until it has been found possible to distinguish experimentally between the two possibilities.) This would account for the different shapes of the curves on the two sides of the point of maximal viscosity. It would further account for the observation that with increasing alkalinity the curve approximates more and more closely to the hyperbolic type, for it is recognised that a protein gel resembles in many of its properties a true solution much more nearly than does a protein sol; this is presumably due to the facts that the micellae are highly hydrated and that the volume occupied by the intermicellar liquid is negligible in comparison with that occupied by the micellae.

Summary.

The isoelectric points of the haemocyanins from *Cancer* and *Homarus* are at p_H 4·7; that of haemocyanin from the snail (*Helix pomatia*) is at p_H 5·3.

The viscosity of solutions of haemocyanin from *Cancer* passes through a

maximum at p_H 7·3; at the same p_H the affinity of the pigment for oxygen passes through a minimum. Viscosity determinations with solutions of haemocyanin from *Homarus* suggest that a similar relationship holds in the case of this pigment.

The above results taken in conjunction with the shape of the oxygen dissociation curves determined at different p_H lead to the following conclusions. The affinity of the pigment for oxygen is probably at a maximum at the isoelectric point; alternatively, maximal affinity occurs on the acid side of the isoelectric point at a p_H corresponding with maximal ionisation of the protein as cation. It is not at present possible to differentiate between these two possibilities owing to an abnormal change which haemocyanin from *Cancer* undergoes at p_H 4. As the pigment ionises as anion its affinity for oxygen decreases, passes through a minimum at a p_H at which ionisation is at a maximum, and increases again as the ionisation is repressed by the presence of higher concentrations of alkali. Finally, as the p_H approaches more and more closely to that at which gelation occurs, the dissociation curve approximates more and more closely to the type to be expected if the combination obeyed the law of mass action. The shape of the curve is controlled by two factors: the charge on the micellae, and their state of hydration.

The expenses of this investigation, which has been carried out during the tenure, by one of us, of a Carnegie Teaching Fellowship, have been met by grants from the Government Grant Committee of the Royal Society and the Moray Research Fund of this University.

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LXXVII. THE EFFECT OF HALOGEN SALTS ON PEPTIC DIGESTION.

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In a previous paper [Clifford, 1925] the effects of the halogen salts of sodium, potassium, ammonium, and calcium on salivary digestion were described. These salts were found to vary in their action on ptyalin, some delaying digestion, others being inert, and some increasing the speed of reaching the achromic point.

It was thought advisable to continue the investigation with other enzymes, and the experiments to be described give results obtained as to the effects of halogen salts on the action of pepsin. Very little work appears to have been done on the influence of salts on peptic digestion. Hamburger and Halpern [1916] found that sodium chloride in "high concentration" only (0·25 %) inhibited peptic activity, whilst in "low concentration" (0·170 %) it accelerated it. They obtained similar results with sodium phosphate.

Rona and Kleinmann [1924] investigated the effects of the chlorates, nitrates and sulphates of various metals, and using a nephelometric method found that all the salts hastened peptic digestion if the p_H of the solution were high and the concentration of the salts low, whilst the reverse was the case if the p_H were low or the salts more concentrated. Cole [1926, p. 248] says "neutral salts inhibit the action of pepsin, this being in marked contrast to the influence of sodium chloride on ptyalin." Bayliss [1924] states, as a generalisation on ferments, that "neutral salts also have an influence, as a rule, of a favourable kind."

Experimental method.

The end-point, or any specific intermediate point of ordinary peptic digestion is difficult to ascertain, since there is no ready method of estimating the amount of peptone and polypeptides produced. Precipitation methods, such as Fuld's described by Cole [1926, p. 252], could not be used in experiments involving the use of halogen salts, for such salts would precipitate part of the edestin and obscure the end-point of the experiment. Finally it was decided to use the clotting of milk, recommended by Cole [1926, p. 249], as a measure of peptic activity, since it is a definite and easily seen change.

The actual experimental method was as follows.

10 cc. milk were pipetted into each of several test-tubes, standard solutions of the halogen salts in other tubes, and for control experiments distilled water in another tube. These solutions were brought to 37° in a water-bath. Then to one of the milk tubes 2 cc. of the distilled water or salt solution were added, and 2 cc. of the pepsin solution. The tube was returned to the bath, and a stop-watch started. At the moment when clotting began the watch was stopped. In this way a definite end-point could be seen and the effect of the salts on the rate of coagulation noted. The strength of the pepsin solution was adjusted till 10 cc. milk, 2 cc. water, and 2 cc. pepsin clotted in $1\frac{1}{2}$ to 3 minutes.

To determine the moment of clotting, the test-tube was removed from the water-bath, tilted gently and replaced as rapidly as possible. This produced a thin film of milk which trickled down the side of the tube. The tilting was repeated every 30 seconds. When clotting was incipient the film became flocculent instead of homogeneous, and the whole tube of milk became solid about 2–5 seconds after the formation of the clotted film.

After the first tube had been roughly tested in this manner, the next tube was left in the bath till 15 seconds before the anticipated time of coagulation. It was then tilted as described, returned to the bath, and generally the film had not time to fall before becoming flocculent. If this were not the case, the tube was again tilted, and the start of coagulation looked for in the new film. In this manner cooling with consequent retardation of enzyme action was minimised.

The figures given in the following tables for each strength of each salt represent the average of results obtained from six tubes. The greatest difference in any one set of six was 8 seconds.

Actual figures for one of the standard experiments with 2 cc. water are 112, 108, 108, 110, 111, 112 seconds.

Two separate batches of commercial pepsin scales were used in these experiments, one requiring a 0·25 % solution, and the other a 0·4 %. At least two separate samples of each halogen salt were also tested.

The concentration of halogen salts varied from 2 cc. of 2*M* solution to 2 cc. of 0·062 *M* solution, each successive dilution being 50 % of the previous one.

Since these 2 cc. were in a bulk of 14 cc. liquid, the actual experimental concentration was $\frac{1}{7}$ of the weighed halogen solution.

Experimental results.

Fluorides.

Conc. *M*	NaF	KF	NH_4F
0·28	2 hrs. 30 mins. } No clot	2 hrs. 15 mins. } No clot	3 hrs. 35 mins. } No clot
0·14	2 „ 30 „	2 „ 15 „	3 „ 35 „
0·07	2 „ 30 „	2 „ 15 „	3 „ 35 „
0·35	1 min. 43 secs.	2 „ 15 „	3 „ 35 „
0·0175	1 „ 23 „	16 mins. 25 secs.	1 min. 10 secs.
0·00875	1 „ 30 „	0 „ 31 „	—
0·0 (control) Standard with water	1 „ 46 „	1 „ 46 „	1 min. 46 secs.

From these results it is seen that these soluble fluorides completely inhibit the clotting of milk by pepsin in concentrations greater than 0·035 *M* for the Na salt and 0·0175 *M* for the K and NH_4 salts. In the lower concentrations the action is reversed and the rate of coagulation hastened by the fluorides.

Chlorides.

Conc. *M*	LiCl	NaCl	KCl	NH_4Cl
0·28	1 min. 7 secs.	1 min. 22 secs.	1 min. 50 secs.	0 min. 40 secs.
0·14	1 „ 0 „	0 „ 57 „	1 „ 27 „	0 „ 34 „
0·07	0 „ 53 „	0 „ 49 „	1 „ 0 „	0 „ 30 „
0·035	0 „ 56 „	0 „ 50 „	1 „ 2 „	0 „ 32 „
0·0175	1 „ 3 „	1 „ 0 „	1 „ 2 „	0 „ 32 „
0·00875	1 „ 12 „	—	1 „ 53 „	1 „ 30 „
0·0 (control)	1 „ 45 „	1 min. 45 secs.	2 „ 6 „	2 „ 6 „

These figures show that the chlorides hasten the rate of clotting, the ammonium salt being the most active.

There is also an optimum concentration for this reaction ranging between 0·07 molar and 0·018 molar, above and below which the acceleration is less marked.

As far as this phase of digestion is concerned, there is certainly no inhibitory effect exerted by NaCl on pepsin in a concentration of approximately 1·5 %, though Hamburger and Halpern [1916] state that a concentration of NaCl above 0·25 % inhibits peptic activity.

Conc. *M*	$MgCl_2$	$CaCl_2$	$BaCl_2$
0·28	0 min. 41 secs.	Instantaneous	Instantaneous
0·14	0 „ 4 „	„	„
0·07	Instantaneous	„	„
0·035	„	„	„
0·0175	„	„	„
0·00875	„	„	„
0·0 (control)	2 mins. 40 secs.	1 min. 47 secs.	3 mins. 0 secs.

The salts of this group, as expected from the well-known action of $CaCl_2$ on clotting reactions, produced a great increase in the rate of coagulation. This increase was much less marked with $MgCl_2$ in the higher concentrations, though in smaller concentration it equalled that given by the Ca and Ba salts.

Bromides.

The results obtained with the bromides are given in the curves in Fig. 1. They are similar to those obtained with chlorides.

The existence of an optimum concentration, however, is more strongly marked (Fig. 1) where it can be seen that with NaBr and KBr a concentration of 0·28 *M* causes definite retarding action. With KBr the hastening is only feebly shown with a concentration as low as 0·0175 *M*. Again the NH_4 salt is the most active.

Conc. *M*	$MgBr_2$	$CaBr_2$	$BaBr_2$
0·28	1 min. 17 secs.	Instantaneous	Instantaneous
0·14	0 „ 9 „	„	„
0·07	0 „ 4 „	„	„
0·035	0 „ 3 „	„	„
0·0175	0 „ 3 „	„	„
0·00875	—	„	„
0·0 (control)	1 min. 54 secs.	1 min. 54 secs.	1 min. 54 secs.

These results are similar to those with the corresponding chlorides, viz. a marked hastening which is more marked with Ca and Ba than with Mg. Again the higher concentrations of the Mg salt are less powerful than the lower.

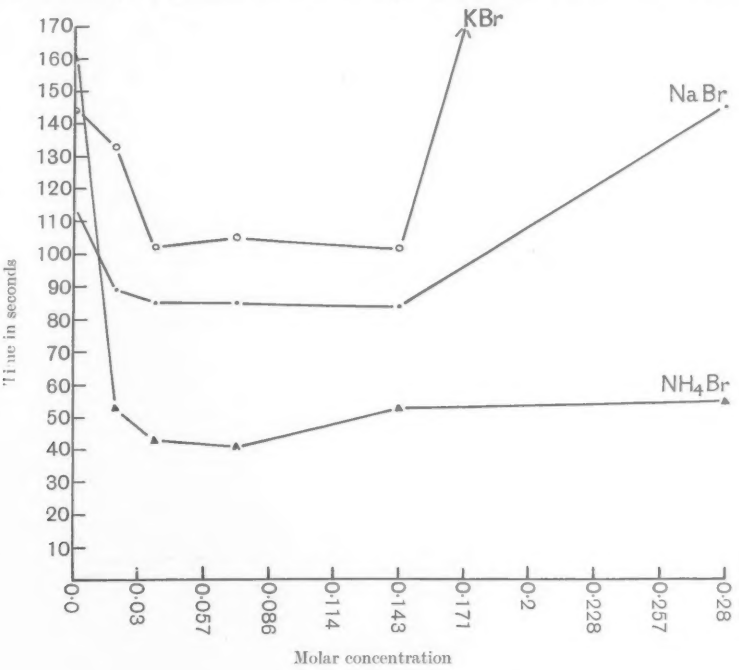

Fig. 1.

Iodides.

Conc. *M*	LiI	NaI	KI	NH_4I
0·28	3 hrs. 0 mins. } No	2 hrs. 20 mins. } No	1 hr. 54 mins. } No	5 mins. 7 secs.
0·14	3 „ 0 „ } clot	2 „ 20 „ } clot	1 „ 54 „ } clot	3 „ 8 „
0·07	3 „ 0 „	2 „ 20 „	1 „ 54 „	2 „ 34 „
0·035	41 mins.*	3 mins. 36 secs.	3 mins. 18 secs.	1 „ 26 „
0·0175	2 mins. 15 secs.	1 „ 54 „	1 „ 42 „	1 „ 29 „
0·00875	1 „ 43 „	—	—	—
0·0 (control)	1 „ 43 „	1 min. 54 secs.	1 min. 54 secs.	2 mins. 55 secs.

* This clot was very fine in each of the six tubes. The milk never became solid, but showed a distinctly granular appearance.

This series shows marked differences. The iodides of Li, Na and K completely stop the coagulative action of pepsin on milk up to a concentration of 0·07 *M*, whilst the NH_4 salt causes a comparatively slight retardation up to a concentration of 0·14 *M*, and then accelerates the rate.

The Li, Na, and K salts in low concentrations appear to have no effect on the rate of coagulation of milk by pepsin.

Conc. *M*	MgI_2	CaI_2	BaI_2
0·28	0 mins. 51 secs.	Instantaneous	Instantaneous
0·14	0 „ 14 „	„	„
0·07	0 „ 9 „	„	„
0·035	0 „ 10 „	„	„
0·0175	0 „ 11 „	„	„
0·00875	—	„	„
0·0 (control)	3 mins. 0 secs.	3 mins. 0 secs.	3 mins. 0 secs.

This series is similar to the other two alkaline earth series. All show a marked hastening influence on coagulation which is much less pronounced with the Mg salt than with the salts of Ca and Ba.

From the results with the Na, K and NH_4 series, it seems that the halide radicle is the primary factor in altering the coagulation rate. The ammonium radicle, however, influences the reaction in a favourable manner.

With the Mg, Ca, Ba series the quickening influence is a function of the metallic radicle, Mg being by far the least potent.

These results are dependent on concentration, for in any series a high concentration of salt was accompanied by an increase in the time for coagulation to occur, except with salts of Ca and Ba where the influence of the metallic radicle is overpowering in any of the strengths used.

Summary.

(1) The clotting of milk by pepsin is greatly affected by the addition of halogen salts.

(2) The reaction is hastened by the chlorides and bromides of Na, K and NH_4, the NH_4 salts having the strongest effect.

(3) There is an optimum concentration of Na, K and NH_4 chlorides and bromides which lies between 0·07 and 0·018 *M*.

(4) The iodides and fluorides of Na, K and NH_4 on the whole retard or inhibit the action of pepsin in coagulating milk.

(5) The inhibitory action of NH_4I is very weak compared with that of NaI and KI.

(6) This retarding action is also a function of concentration, since at low concentrations the iodides do not affect the rate, whilst the fluorides actually hasten it.

(7) The NH_4 radicle favours the coagulation of milk by pepsin.

(8) In the presence of Mg, Ca and Ba halides there is a great increase in the rate of coagulation of milk by pepsin. This action is weakest with the Mg salts.

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LXXVIII. STUDIES IN CARBOHYDRATE METABOLISM.

III. THE INFLUENCE OF DIHYDROXYACETONE UPON THE RESPIRATORY METABOLISM AND UPON THE INORGANIC PHOSPHATE OF THE BLOOD.

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Two divergent views are at present held regarding the path by which dihydroxyacetone is metabolised. According to one view—that of Campbell, Fletcher, Hepburn and Markowitz [1926]—it is first of all entirely converted into glucose which is then metabolised in the ordinary way.

According to the second view, dihydroxyacetone may be in part directly oxidised, although, in the diabetic, it may be more or less completely converted into glucose according to the severity of the condition, whilst even in the normal individual a certain amount may go into glucose, particularly after large doses [Kermack, Lambie and Slater, 1927]. Indirect evidence pointing to direct utilisation is afforded by the observation that the blood-sugar curves, both in diabetic and in normal subjects, after administration of dihydroxyacetone (50 g.) not only do not rise to so great a height as after an equivalent amount of glucose, but return more rapidly to the original level [Isaac and Adler, 1924; Rabinowitch, 1925; Kermack, Lambie and Slater, 1926; Mason, 1926, 1]. As regards recovery from insulin hypoglycaemia, Kermack, Lambie and Slater [1927] could find no significant difference between the time taken to remove the symptoms after administration of the same dose of glucose and of dihydroxyacetone. Moreover, after dihydroxyacetone recovery sometimes occurred in spite of the fact that the blood-sugar had apparently not risen to the "convulsion level." More direct evidence, suggestive of direct utilisation, was furnished by the experiment upon the decerebrated cat with the liver excluded from the circulation, in which it was shown that the muscles removed dihydroxyacetone from the blood much more rapidly than glucose when the two substances were transfused at an equal rate. This experiment does not, however, tell us whether the dihydroxyacetone which is removed by the muscles is oxidised or whether it is merely stored in some form.

In order to settle the question of direct oxidation it would be necessary to show that dihydroxyacetone could raise both the respiratory quotient and

the oxygen consumption more rapidly than glucose. If it could be demonstrated that these changes in the respiratory metabolism occur in the diabetic after administration of dihydroxyacetone under conditions where they are absent or less marked after glucose, the evidence in favour of direct oxidation would be further strengthened.

Mason [1926, 2] has found that dihydroxyacetone when administered by the mouth does, as a matter of fact, raise the respiratory quotient and the metabolic rate more rapidly and more markedly than an equal amount of glucose, and this occurred both in diabetic and in normal individuals. A possible objection, may, however, be raised to the conclusions drawn from his experiments owing to the consideration that the differences observed may have been due to dihydroxyacetone being more rapidly absorbed from the alimentary canal than glucose. It was therefore decided to repeat Mason's observations and to compare the effects of dihydroxyacetone and glucose upon the metabolism after intravenous administration. A few similar experiments were also performed with laevulose. In order to see whether the observations of Campbell, Fletcher, Hepburn and Markowitz regarding the identical behaviour of the inorganic blood-phosphate curves after administration of glucose and dihydroxyacetone could be confirmed, these were followed simultaneously with those of the respiratory metabolism and the blood-sugar.

Experimental.

In all experiments the subject was in the post-absorptive condition. The expired air was collected in 6-minute samples, using the Douglas bag method, and was analysed by means of Haldane's apparatus. The basal metabolic rate was first determined and a sample of blood for blood-sugar, blood-dihydroxyacetone and blood-inorganic phosphate was taken. The blood-sugar was determined by MacLean's method, the inorganic phosphate by Briggs' [1922] modification of the Bell and Doisy method and dihydroxyacetone by Campbell and co-workers' [1926] method. After administration of the carbohydrate, similar determinations were made at intervals.

In the first series of experiments oral administration was employed.

Oral administration.

Exp. 1 (*a*). Respiratory metabolism and curves of blood-sugar and blood-phosphate in normal student after ingestion of 50 g. glucose.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar mg. %	Blood-inorganic phosphate mg. %
0	− 3	1·484	0·81	31·5	94	3·629
Glucose 50 g.						
18	± 0	1·486	0·91	32·1	111	3·629
31	+10	1·712	0·75	35·0	109	3·605
46	+10	1·700	0·76	34·0	100	3·358
61	+ 4	1·663	0·79	34·5	87	3·388
91	+ 4	1·695	0·84	33·25	84	3·750
128	+ 1	1·564	0·76	32·0	75	3·308
173	−13	1·378	0·8	27·85	87	3·358

Exp. 1 (*b*). Same as preceding after ingestion of 50 g. dihydroxyacetone.

Time mins.	Metabolic rate	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar mg. %	Blood-dihydr. mg. %	Blood-inorganic phosphate mg. %
0	+ 3	1·643	0·75	28·32	86	0	3·432
Dihydroxyacetone 50 g.							
15	+21	1·779	1·10	39·36	86	5	2·848
20	+35	1·976	1·10	46·50	105	30	2·526
44	+31	1·941	1·10	48·90	106	49	2·368
67	+10	1·614	1·10	45·21	87	25	2·586
97	+ 4	1·469	1·10	40·56	68	5	2·884
127	+13*	1·909	0·707	35·10	76	5	2·960
157	− 3	1·593	0·67	35·10	68	0	2·960

* Mask readjusted.

Exp. 2 (*a*). Respiratory metabolism and curves of blood-sugar and blood-phosphate in normal student after ingestion of 50 g. glucose.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar mg. %	Blood-inorganic phosphate mg. %
0	− 1	1·750	0·81	37·2	117	3·243
Glucose 50 g.						
19	+ 2	1·789	0·72	36·0	123	3·236
38	+18	2·092	0·76	41·6	113	3·000
47	+15	1·957	0·85	43·5	110	2·925
59	+19	2·091	0·73	42·5	76	2·703
88	+13	2·005	0·7	40·2	67	2·928
116	+ 7	1·865	0·76	37·6	80	2·756

Exp. 2 (*b*). Same as preceding after 50 g. laevulose.

Time mins.	Metabolic rate	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar mg. %	Blood-laevulose mg. %	Blood-inorganic phosphate mg. %
0	+15	1·620	0·8	40·5	110	0	2·008
Laevulose 50 g.							
25	+26	1·817	0·72	40·0	98	25	1·825
39	+20	1·716	0·75	39·0	69	0	1·759
49	+ 1	1·409	0·84	35·5	—	—	—
68	+ 8	1·460	0·82	35·0	—	—	—
95	+10	1·555	0·85	36·0	70	0	1·715

Exp. 3 (*a*). Respiratory metabolism and curves of blood-sugar and blood-phosphate in a diabetic patient after ingestion of 50 g. glucose.

Time mins.	Metabolic rate	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar mg. %	Blood-inorganic phosphate mg. %
0	+18	1·339	0·60	26·35	168	3·571
Glucose 50 g.						
15	− 7	1·051	0·66	23·10	192	3·562
34	− 3	1·149	0·62	23·50	204	3·556
49	+15	1·317	0·59	24·25	251	3·571
69	+11	1·259	0·63	24·25	295	2·777
109	+ 7	1·200	0·65	24·25	341	2·900
149	+ 5	1·192	0·63	23·75	312	3·409
190	− 5	1·091	0·56	24·35	255	3·333

Exp. 3 (*b*). Same as preceding after ingestion of 50 g. dihydroxyacetone.

Time mins.	Metabolic rate	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar mg. %	Blood-dihydr. mg. %	Blood-inorganic phosphate mg. %
0	+ 2	1·147	0·65	24·50	187	0	3·1846
Dihydroxyacetone 50 g.							
24	+ 9	1·173	0·85	26·65	206	60	2·568
46	+50	1·615	0·80	37·39	230	70	2·272
65	+29	1·423	0·71	32·50	270	55	2·343
98	+14	1·285	0·66	28·43	249	25	2·922
132	+ 2	1·150	0·65	23·00	231	10	2·445
160	+ 5	1·182	0·63	23·61	243	5	2·445

Exp. 3 (*c*). Same as preceding after ingestion of 50 g. laevulose.

Time mins.	Metabolic rate	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar mg %	Blood-inorganic phosphate mg. %
0	± 0	1·227	0·57	24·15	133	3·703
Laevulose 50 g.						
15	− 7	1·234	0·63	24·25	173	3·703
30	+16	1·370	0·69	27·5	202	3·750
46	+15	1·367	0·68	28·0	222	3·571
60	+18	1·390	0·72	29·75	222	3·845
90	+ 5	1·227	0·68	25·0	222	?
123	+ 2	1·140	0·99	25·0	202	3·488

Exp. 4 (*a*). Respiratory metabolism and curves of blood-sugar and blood-phosphate in a diabetic patient after ingestion of 50 g. glucose.

Time mins.	Metabolic rate	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar mg. %	Blood-inorganic phosphate mg. %
0	−15	1·063	0·79	24·90	152	3·304
Glucose 50 g.						
51	± 0	1·360	0·6	27·34	253	3·300
82	+ 6	1·371	0·62	28·50	400	2·905
143	−23	0·801	0·51	22·50	444	3·321

Exp. 4 (*b*). Same as preceding after ingestion of 50 g. dihydroxyacetone.

Time mins.	Metabolic rate	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar mg. %	Blood-dihydr. mg. %	Blood-inorganic phosphate mg. %
0	−12	1·134	0·65	30·50	145	5	3·289
Dihydroxyacetone 50 g.							
12	−11	1·116	0·74	30·50	190	25	2·952
33	+44	1·818	0·74	46·25	202	55	2·686
53	+37	1·819	0·73	48·25	253	35	2·343
73	−10	1·159	0·66	28·20	—	—	—
140	−10	1·159	0·65	28·75	222	5	2·393
179	−20	1·047	0·61	27·50	210	5	2·678

Intravenous administration.

In the next series the substances were injected intravenously.

The solutions for intravenous injection were made by dissolving 25 g. glucose or dihydroxyacetone in physiological saline in the cold and making up the volume to 50 cc. Sterilisation of the solution was effected by passing it through a Seitz germicide (EK) filter No. 6, using special pads impermeable to micro-organisms. 40 cc. of the fluid, previously warmed to 37°, were injected into a superficial vein at the bend of the elbow, the injection being made at a uniform rate and occupying a period of exactly 5 minutes. Strong solutions were employed in order to avoid excessive blood-dilution, and to minimise any disturbing effects upon metabolism occasioned by the injection of large volumes of fluid. Great care must, however, be exercised in the use of such hypertonic solutions owing to the risk of producing venous thrombosis. While the injection was in progress there occurred, with dihydroxyacetone, an obvious increase in the depth and frequency of the respiratory movements. At first it was thought that this might have been due to the direct stimulation of the respiratory centre by dihydroxyacetone, or to something of the nature of a "specific dynamic action." The analyses of 3-minute samples of expired air taken during this period show, however, that the increased breathing was accompanied by a rise in the total metabolism, in the oxygen consumption and in the R.Q., indicating that the carbohydrate itself was being rapidly metabolised. These phenomena were not observed in the case of glucose.

Exp. 5 (*a*). Respiratory metabolism and curves of blood-sugar and blood-phosphate in a normal student after intravenous injection of 20 g. glucose.

Time mins.	Metabolic rate	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar mg. %	Blood-inorganic phosphate mg. %
0	+ 7	1·766	0·73	37·5	85	3·515
1–5 Glucose 20 g.						
16	+ 8	1·760	0·79	43·35	156	3·260
31	+24	2·441	0·80	43·75	112	3·571
44	+19	1·887	0·82	42·5	75	4·40
58	+17	1·904	0·70	40·0	67	3·571
74	+16	1·927	0·78	41·8	67	3·375
103	+15	1·898	0·73	41·35	76	4·40
136	+ 8	1·774	0·72	37·20	73	—

Exp. 5 (*b*). Same as preceding after intravenous injection of 20 g. dihydroxyacetone.

Time mins.	Metabolic rate	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar mg. %	Blood-dihydr. mg. %	Blood-inorganic phosphate mg. %
0	– 7	1·59	0·83	39·35	90	15	3·125
1–5 Dihydroxyacetone 20 g.							
1–3	+15	1·94	0·87	42·00	—	—	—
3–6	+50	2·51	0·92	61·50	—	—	—
17	+49	2·54	0·86	57·00	92	20	2·240
27	+36	2·35	0·81	51·00	93	15	2·312
40	+12	1·91	0·84	47·00	82	15	2·351
52	+ 7	1·85	0·80	46·50	70	15	2·700
67	+ 3	1·8	0·76	40·25	69	15	2·725

Exp. 6 (*a*). Respiratory metabolism and curves of blood-sugar and blood-phosphate in a diabetic patient after intravenous injection of 20 g. glucose.

Time mins.	Metabolic rate	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar mg. %	Blood-inorganic phosphate mg. %
0	+ 1	1·239	0·5	25·5	168	3·750
1–5 Glucose 20 g.						
12	− 1	1·188	0·5	25·0	245	3·685
25	+ 8	1·381	0·51	27·0	236	3·597
40	+10	1·274	0·62	28·0	205	3·456
60	− 4	1·220	0·56	27·0	212	3·658
68	− 4	1·125	0·56	25·0	195	3·658
105	− 4	1·115	0·52	25·0	167	3·428

Exp. 6 (*b*). Same as preceding after intravenous injection of 20 g. dihydroxyacetone.

Time mins.	Metabolic rate	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar mg. %	Blood-dihydr. mg. %	Blood-inorganic phosphate mg. %
0	+ 2	1·227	0·55	27·50	202	80·0	3·333
1–5 Dihydroxyacetone 20 g.							
1–3	+46	1·735	0·59	35·20	—	—	—
3–6	+59	1·792	0·85	41·00	—	—	—
16	+56	1·749	0·88	41·75	253	35·0	2·884
33	+21	1·687	0·88	34·00	246	27·5	2·812
47	−10	1·030	0·73	29·00	246	20·0	2·585
63	− 6	1·071	0·79	31·00	222	15·0	2·585
95	± 0	1·201	0·60	29·30	214	12·0	3·409?
126	−18	0·952	0·77	28·25	200	10·0	2·884

Discussion.

The above experiments amply confirm the results of Mason regarding the differences which occur in the respiratory metabolism following administration of dihydroxyacetone and of glucose when the substances are given by the mouth. In fact, by taking air and blood samples at shorter intervals, the more rapid rise in the total metabolism, in the oxygen consumption and in the respiratory quotient is even more strikingly shown. Thus, within 15 minutes of the ingestion of 50 g. dihydroxyacetone by the normal individual, a rise of about 18 % in the total metabolism was observed, whilst after a similar amount of glucose a rise of only 3 % was obtained. Exp. 1 (*a*) is exceptional in that the respiratory quotient, high to begin with, rose to a maximum 15 minutes after the administration of glucose, but there was no corresponding rise in the oxygen consumption or in the metabolic rate. Overbreathing is the probable explanation of this result. In all other experiments, both in diabetics and normals, the R.Q. attained its maximum about 45 minutes after administration of glucose, as did also the total metabolism and the oxygen consumption. On the other hand, after dihydroxyacetone the various maxima were reached in 20 to 30 minutes. After this there was a rapid fall in all values. The experiments in which intravenous injection of this substance was employed show that more rapid absorption from the intestine is not sufficient to account for the observed results. Thus, after 20 g. glucose intravenously, the total

metabolism, oxygen consumption and respiratory quotient gradually rose, reaching their maximum in approximately half an hour. Simultaneously with the rise in the respiratory metabolism, the blood-sugar fell and had regained the normal level or fallen below it about the time that the metabolism was at its height. With dihydroxyacetone, on the other hand, the rise in the total metabolism, the oxygen consumption and the respiratory quotient was not only more marked than with glucose, but was immediate, attaining its maximum about the time that the injection was completed, after which there was a fall *pari passu* with the disappearance of dihydroxyacetone from the blood. The maximal increase in the total metabolism of normal individuals after glucose was about 16 %, while after dihydroxyacetone the metabolism rose to 57 % above the basal level. In the diabetic, the rise in the metabolism after glucose was much smaller than in the normal individual. Thus, in Exp. 6 (*a*) there was an increase of not more than 9 % in the total metabolism after 20 g. glucose intravenously, the respiratory quotient and oxygen consumption also showing only a slight rise. On the other hand, after injection of a similar amount of dihydroxyacetone, the resultant increase in metabolism was comparable to that met with in the normal individual—namely 57 %—the oxygen consumption and respiratory quotient showing a corresponding rise. Dihydroxyacetone, therefore, produced a much greater increase in the metabolism in the diabetic than did glucose in the normal (cf. Exps. 5 (*a*) and 6 (*b*)).

These facts would seem to indicate that dihydroxyacetone can either be directly and immediately oxidised or very rapidly changed into some substance —possibly an active form of dihydroxyacetone—which is oxidised with great ease. Glucose, on the other hand, would appear gradually to undergo some transformation prior to its oxidation. It is evidently this transformation which the diabetic has difficulty in effecting.

The above observations seem to be in accord with the hypothesis advanced in a previous paper [Kermack, Lambie and Slater, 1927]—namely, that the essential feature of insulin action is to facilitate the transformation of glucose into dihydroxyacetone, which is then directly oxidised.

Although dihydroxyacetone may be converted into glucose to a greater or lesser extent in the diabetic, especially after repeated small doses by the mouth, the sudden injection of a large quantity intravenously would cause a certain amount to be rapidly oxidised, thus raising the metabolism in the manner above described, before any considerable amount would have time to be converted into glucose.

This more rapid oxidation of dihydroxyacetone in the animal body is paralleled by its more ready oxidation *in vitro*, as compared with glucose. Thus Wind [1925] has found that in neutral phosphate solution it takes up atmospheric oxygen much more readily than laevulose, while glucose takes up hardly any oxygen at all. Experiments, which will be reported by one of us in detail in a future communication, have been performed which show that, in neutral phosphate solution at p_H 7·4 and 37°, permanganate is much more

rapidly decolorised by dihydroxyacetone than by glucose and that dihydroxyacetone reduces methylene blue under anaerobic conditions whereas glucose does not. The latter observation is of interest in connection with the experiments of Ahlgren [1924], who found that the muscles of depancreatised animals, unlike those of normal animals, were unable to convert glucose into a hydrogen donator as tested by the methylene blue method, whereas addition of insulin restored the capacity to do so. Evidently some intermediary metabolite must have been formed from glucose under the influence of insulin which could act as a hydrogen donator. Dihydroxyacetone, which is a suggested intermediary, could at least fulfil this rôle.

It is now well established that increased metabolism of carbohydrates, whether brought about by ingestion of glucose or by injection of insulin, is accompanied by a fall in the inorganic phosphate of the blood, due in all probability to the formation of phosphoric acid esters of carbohydrate [Wigglesworth, Woodrow, Winter and Smith, 1923; Harrop and Benedict, 1924]. In view of this close connection between phosphate and carbohydrate metabolism together with the differences above described in the respiratory metabolism after administration of glucose and of dihydroxyacetone, there might naturally have been expected to be corresponding differences in the phosphate metabolism. Our protocols show that this is indeed the case. The curves of inorganic blood-phosphate follow closely those of the respiratory metabolism; the fall in inorganic blood-phosphate is more marked and more rapid after dihydroxyacetone than after glucose whether administered by the mouth or intravenously. Moreover, in the diabetic, where very little drop in the inorganic phosphate occurred after glucose, a marked fall followed the administration of dihydroxyacetone. It is difficult to reconcile these findings with those of Campbell, Fletcher, Hepburn and Markowitz [1926]. The fall in the inorganic blood-phosphate is not accounted for by increased excretion in the urine, since it was immediate after intravenous injection of dihydroxyacetone, while in some experiments little if any urine was eliminated during the whole period of the experiment. In other experiments the elimination of phosphate in the urine varied with the urine volume, rather than with the concentration of inorganic blood-phosphate.

These results would appear to indicate that administration of dihydroxyacetone causes the more rapid formation of a phosphoric acid ester than does the administration of glucose. The extremely rapid fall in inorganic phosphate following intravenous injection suggests that dihydroxyacetone may be directly esterified, forming a triosephosphate. Raymond [1925] and Kluyver and Struyk [1926] have proposed schemes in which triosephosphate figures as an intermediary in carbohydrate metabolism, without, however, bringing forward any experimental evidence in support of their views. Glucose, as suggested by Euler [1925] and Meyerhof [1926], is represented as being esterified to hexosemonophosphate, and the latter splits into one molecule of triose and one molecule of triosephosphate. Two molecules of triosephosphate then condense

to form one molecule of hexosediphosphate. This would seem to account more readily for the formation of hexosediphosphate than the explanation offered by Meyerhof, namely, that it is formed by the splitting of two molecules of hexosemonophosphate into hexosediphosphate (1 mol.) and lactic acid (2 mols.). It would be premature to speculate upon the nature or fate of the phosphoric esters actually involved in these processes, as this is a matter for further investigation, but the above experiments serve to draw attention to the possible importance of esters of dihydroxyacetone.

The experiments with laevulose show that this substance is utilised somewhat more easily than glucose, but not nearly as readily as dihydroxyacetone. Its effects upon metabolism appear to be intermediate between the two, but more closely approximating to those of glucose. In experiments upon the normal subject (including some not recorded in the above protocols) the R.Q. after oral administration of 50 g. dihydroxyacetone rose above unity, indicating that fat was being formed from carbohydrate. Respiratory quotients above unity were not obtained with either glucose or laevulose in the doses employed, namely, 50 g. Benedict and Carpenter [1918] have, however, shown that with 100 g. or after repeated administration of smaller quantities, respiratory quotients above unity may be obtained with laevulose but not with glucose. Dihydroxyacetone can therefore produce these high respiratory quotients with smaller doses than laevulose.

Only a small number of experiments upon the respiratory metabolism in diabetics following administration of laevulose have been recorded above, and these show that, in the doses employed, the effects were not very different from those obtained with glucose. It has, however, been demonstrated by numerous investigators [Minkowski, 1890; Lusk, 1915; Verzar, 1914; Tögel and co-workers, 1913; Johansson, Billström and Heijl, 1904] that with larger or repeated doses laevulose produces a much greater rise in the total metabolism and in the R.Q. than similar amounts of glucose. Joslin [1924] has even obtained respiratory quotients of unity or above in diabetics.

Laevulose, like dihydroxyacetone, is not only more easily oxidised in the animal body, but it is a better glycogen former than glucose. This is all the more remarkable in view of the fact that glycogen yields only glucose on hydrolysis. As far as can be ascertained there is no difference between the glycogen formed from glucose and that formed from laevulose [Cremer, 1902; Macleod, 1926]. Neither laevulose nor hexosediphosphate [Harden and Young, 1911; Young, 1911], which is probably a derivative of laevulose, can, therefore, presumably be the immediate precursors of glycogen. There would therefore seem to be some intermediary common to laevulose and to glucose from which glycogen is formed. Another fact pointing to the existence of a common intermediary is that in the diabetic laevulose is excreted for the most part as glucose [Minkowski, 1890]. It has been supposed that in the formation of glycogen from laevulose, the latter is converted into glucose through the common enol form of the hexoses. This would not, however, explain satisfactorily the greater

ease with which laevulose forms glycogen, nor why such a substance as dihydroxyacetone is a better glycogen former than glucose. The simplest hypothesis would seem to be that dihydroxyacetone is the intermediary common to laevulose and glucose and that glycogen is formed from it by condensation and polymerisation. In the diabetic, dihydroxyacetone would be converted into glucose and thus the increased elimination of the latter after administration of laevulose would be accounted for. It at first seems an objection to this assumption that laevulose, in spite of its being more readily oxidised in the animal body, even in diabetes, does not cause recovery from insulin hypoglycaemia as rapidly as does glucose [Noble and Macleod, 1923] or dihydroxyacetone. It is possible, however, that laevulose, being structurally more closely related to dihydroxyacetone than glucose, may be converted into dihydroxyacetone without the aid of insulin, and in this way its more ready utilisation by the diabetic, observed especially after large doses, would be accounted for. On the other hand, insulin may be supposed to be specifically adapted for the conversion of glucose (? and mannose, whose structure closely resembles that of glucose) into dihydroxyacetone, a reaction occurring with difficulty, if at all, without it. In the presence of excess of insulin, however, as in animals suffering from experimental hypoglycaemia, the conversion of glucose into dihydroxyacetone might be even more rapid than the formation of the latter from laevulose. Hence, under these conditions, recovery would be more rapid with glucose than with fructose.

Finally, it may be noted that it is probable that dihydroxyacetone, like other ketoses, can exist in forms other than the stable variety [cf. Fischer and Taube, 1924] and we therefore cannot exclude the possibility that it is in fact a reactive form of the substance which is biologically important.

Summary.

1. Dihydroxyacetone produces, both in diabetics and normals, a more rapid and more marked rise in the respiratory quotient, the oxygen consumption and the total metabolism than does glucose, whether administered intravenously or orally.

2. Dihydroxyacetone can be directly utilised by the diabetic. After intravenous injection of 20 g. over a period of 5 minutes, the immediate rise in the metabolism, respiratory quotient and oxygen consumption is comparable to that obtaining in normal individuals.

3. The fall in the inorganic phosphate of the blood in both diabetics and normals is more rapid and more marked after dihydroxyacetone than after glucose, whether administered intravenously or orally. The curves of inorganic blood-phosphate follow closely those of the respiratory metabolism.

4. Laevulose produces effects intermediate between those of glucose and dihydroxyacetone, but more closely approximating to those of the former when small doses are employed.

5. Respiratory quotients above unity were obtained after oral administration of 50 g. dihydroxyacetone in normals, indicating the formation of fat from carbohydrate.

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LXXIX. THE PURIFICATION AND PROPERTIES OF INSULIN.

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It is a matter of common experience that, whilst the purification of crude insulin to a degree of activity represented by a unit of approximately 0·1 mg. presents few difficulties, attempts to exceed this to any great extent usually result in a considerable loss. The methods employed for this purpose may be grouped into two main classes: (*a*) precipitation at the isoelectric point, and (*b*) the use of protein precipitants.

With the former method it is usual to find that after repeated precipitation the activity is not further increased, moreover, considerable losses accompany each precipitation. This fact is frequently referred to by various workers, *e.g.* Shonle and Waldo [1924], and although in some cases material has been prepared which greatly exceeds the degree of activity represented by the unit mentioned above, such methods of purification are not ideal for the preparation of insulin in quantities sufficient either for clinical use or for experimental purposes, owing to the small fraction of the original number of units which is recovered in the final product after several precipitations.

The use of the second group of methods, whilst it does not generally involve the loss of active material which accompanies isoelectric precipitation, results in little purification beyond the limits referred to above. Moreover, it is frequently impossible to purify by this means certain specimens of insulin, notably those prepared from pancreas in which, owing to insufficient freezing, tryptic action has occurred, so as to obtain material the unit of which is below 1 mg. or even more. The following investigations were undertaken with a view to determining the reasons underlying the failure by these methods.

In order to characterise the group of protein fission products to which insulin belongs it was thought that the application of the recently described method of Wasteneys and Borsook [1924] would prove to be of value. These workers showed that if trichloroacetic acid were added to a solution containing a mixture of proteins and their disintegration products in sufficient quantity to make up the concentration in the solution to 2 %, then proteins and metaproteins alone were precipitated. On applying this method to insulin solutions, it was found that the whole of the potency was precipitated under these con-

ditions. The supernatant fluid from this precipitation was always found to give a further precipitate on the addition of picric acid, even when insulin of considerable purity was used. In order to test the effect of this 2 % trichloroacetic acid precipitation quantitatively it was necessary to devise some method whereby the insulin could be recovered from the trichloroacetic acid precipitate, and it was found that if this were dissolved in dilute acid, the activity was completely precipitated by the addition of sodium chloride. This precipitate was free from trichloroacetic acid, and could easily be converted into the hydrochloride.

In this way, a quantitative recovery of the activity results, together with considerable purification. If a specimen of insulin which has been through this process is subjected to treatment with 2 % trichloroacetic acid again, it is found that the whole is precipitated and that the supernatant fluid yields at most a trace of picrate, and that therefore nothing is to be gained by repeating this treatment. This experiment would show, according to Wasteneys and Borsook, that the active fraction of insulin must be associated with the groups of protein or metaprotein, but the application of these views to the question of the constitution of insulin is discussed later. It is clear, however, that the greater part of the material precipitated by 2 % trichloroacetic acid must consist of proteins or metaproteins, and this suggested that physico-chemical means might be applied to the problem of further purification.

Hardy [1905] showed that at or near their isoelectric point globulins can be dissolved by neutral salt solutions. More recently Cohn [1924] has shown that the degree of such solubility is a characteristic property of each individual protein, and it was thought that the application of this principle to insulin might yield valuable results. Accordingly an isoelectric precipitate of insulin was washed with solutions of potassium chloride, both the material dissolved and the insoluble residue being converted to hydrochloride. Tests upon rabbits demonstrated that the active fraction is relatively insoluble in dilute salt solution, whereas some of the inactive portion dissolves more readily; thus purification has been effected.

Hardy also showed that bivalent and tervalent anions are capable of holding in solution more globulin than are univalent anions. After some preliminary trials it was decided to investigate the action of oxalates and citrates upon insulin, but it was clear that an experiment similar to the one quoted above with sodium chloride could not be performed, because these salts are in themselves weak buffers, the solutions having a p_H of 7 or over, and if the isoelectric precipitate of insulin is washed with a solution of potassium oxalate, then the precipitate dissolves, owing to the fact that the p_H rises above that of the isoelectric point.

The effect of adding a $N/8$ solution of potassium oxalate to a solution of insulin hydrochloride (which is strongly acid) was then observed. On adding such a solution drop by drop it was found that a precipitate began to appear when the p_H had reached about 3·7, and reached a maximum at about p_H 4,

and then the solution began to clear as the p_H rose. The formation of a maximum precipitate between p_H 3·7 and 4·2, instead of at the normal isoelectric point (p_H 4·5–5·0 with this particular material), appeared to be capable of explanation by the work of Michaelis and Szent-Györgyi [1920], who showed that the isoelectric point of certain proteins can be definitely lowered by salts. The experiments described confirm this, in the case of insulin, and the lowering of the isoelectric point is shown to depend upon the concentration of oxalate present. Great purification was obtained in this manner with little or no loss of activity.

In the light of the foregoing experiments it appears that the following explanation may be advanced to account for this phenomenon. The addition of a weak buffer of potassium oxalate slowly raises the p_H of the solution and at the same time lowers the isoelectric point of the insulin until a precipitate is produced. The potassium oxalate holds in solution inactive fractions which will be precipitated during the course of an ordinary isoelectric precipitation.

This type of method has certain objections both from a practical and a theoretical aspect. In the first place, it is difficult to determine the end-point of the titration except by centrifugation after each small addition of oxalate, and, secondly, since the amount of inactive material held in solution must vary with the quantity of oxalate present, it is often impossible to obtain a sufficiently high concentration of oxalate without raising the p_H to a level above the isoelectric point, thus dissolving some of the active portion. A method was therefore devised in which the insulin solution could be added to a buffer solution of suitable p_H value which contained sufficient oxalate to hold the relatively greater quantities of inactive material in solution. It was found that to produce a p_H of 4, the point at which maximum precipitation occurs in the above experiment, 100 cc. of potassium oxalate solution must be added to 20 cc. of oxalic acid solution. The p_H of the solution of insulin hydrochloride was adjusted to 4, and it was then run into two volumes of the above buffer solution with vigorous stirring. A precipitate was produced which was found to contain all the original potency in a much higher concentration, showing that a large amount of inactive material had been removed.

By combining the trichloroacetic acid precipitation, the acid and salt precipitation and the oxalate precipitation, a very efficient purification method was obtained. Similar results are obtained when sodium citrate is used in place of potassium oxalate. The details of these investigations are given in the experimental section.

In the above examples we have employed solutions of approximately p_H 4, since, for the particular specimens of insulin used (isoelectric point at or about p_H 5), this represents the optimum condition. We have observed, however, that many specimens of insulin hydrochloride possess an isoelectric point much higher than that recorded hitherto. This applies more especially to some specimens of material prepared by the acetone-picric acid process, the isoelectric point of which is frequently at p_H 5·9, or even higher. If such solutions

be brought to p_H 5·7 there is often no sign of precipitation. In this case, the oxalate mixture at p_H 4 is clearly too acidic and only a small fraction of the activity is precipitated on mixing the solutions at this hydrogen ion concentration. By first performing an isoelectric precipitation on a portion of the solution and determining the hydrogen ion concentration at maximum precipitation this difficulty can be avoided. In such cases the reactions of the oxalate solution and the insulin solution should be brought to a higher p_H value (approximately 5·0) before mixing, or the original method of running the solution of potassium oxalate into the solution of insulin hydrochloride to maximum precipitation may be employed.

Experimental.

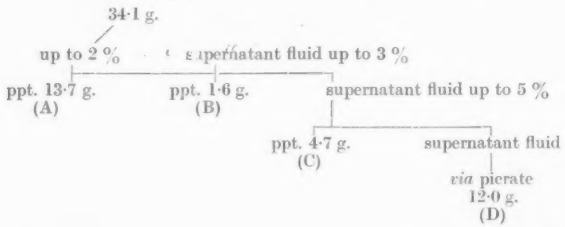
The precipitation of insulin by trichloroacetic acid.

(*a*) *Effect of concentration.* Crude insulin hydrochloride (19·7 g.) was dissolved in water to give a 1·5 % solution. A 10 % solution of freshly distilled trichloroacetic acid was added drop by drop with constant stirring until the concentration of the acid was 1 %. There was no precipitation. A further measured quantity of trichloroacetic acid was added to bring the concentration up to 1·5 %. The precipitate which was obtained was allowed to stand for 2 hours and was then centrifuged as completely as possible. Although this operation was performed at a high speed, the supernatant fluid was still faintly turbid. It was decanted from the precipitate and trichloroacetic acid was added to it to bring the concentration up to 2 %. A further precipitation resulted, and after centrifugation the supernatant fluid was perfectly clear. The precipitates were dissolved in $N/10$ hydrochloric acid and, on the addition of half the volume of saturated sodium chloride solution to each, a precipitate formed. This was collected in the centrifuge, dissolved in water, and the material was obtained from the solution in the usual way by precipitation as picrate and conversion to the hydrochloride [Dudley and Starling, 1924]. The two fractions thus obtained weighed 1·86 and 2·84 g., and standardisation showed that they contained 1230 and 2840 units respectively. The initial material had 4500 units. This experiment shows that by means of the precipitation 15·0 g. of almost completely inactive material had been removed. It is also clear that, whereas a 1·5 % concentration of trichloroacetic acid is insufficient for complete precipitation of the activity, 2 % trichloroacetic acid causes almost the whole of the activity (90 % in this example) to be precipitated. The addition of further quantities of 10 % trichloroacetic acid brought about the precipitation of inactive material.

In the above example very crude insulin hydrochloride was used. Similar experiments with highly purified material have also shown that the activity is completely precipitated by 2 % trichloroacetic acid.

(*b*) *Fractional precipitation with trichloroacetic acid.* An experiment performed in a manner similar to that described above was carried out, using

concentrations of trichloroacetic acid of 2, 3 and 5 %. The precipitates in each case were converted to hydrochloride. The results are shown in the following table. The initial weight of material (34·1 g.) contained 34,000 units. Precipitate A below, the unit of which proved to be 0·44 mg., contained 31,000 units.



Of these fractions A is the only one which behaves as a typical protein or metaprotein, *e.g.* it is precipitated at the isoelectric point, completely precipitated by half saturation with ammonium sulphate and by copper sulphate. B gives only a very faint turbidity at the isoelectric point, whilst solutions of C and D remain perfectly clear, and all three yield a further precipitate on full saturation with ammonium sulphate and are not precipitated by copper sulphate. The precipitates of B, C and D, obtained on adding potassium ferrocyanide and acetic acid, clear on warming, indicating the presence of primary proteose in these fractions.

The distribution of amino- to total nitrogen in these fractions is shown in Table I. It will be seen that there is a gradual rise in the ratio of amino- to total nitrogen with each fraction.

Table I.

Trichloroacetic acid	Amino-N %	Total N %	$\frac{\text{Amino-N}}{\text{Total N}}$ %
(A) Up to 2 %	0·82	14·06	5·8
(B) 2 to 3 %	0·94	14·65	6·4
(C) 3 to 5 %	0·98	14·80	6·6
(D) Not precipitated by 5 % trichloroacetic acid	1·41	15·00	9·4

Solubility of the isoelectric precipitate in salt solutions.

(1) *Potassium chloride.* Qualitative experiments having shown that the inactive fractions, separated from insulin during the course of purification by various means, tend to dissolve much more readily when potassium and sodium chloride solutions are added to their isoelectric precipitates than does the active portion, the following roughly quantitative experiment was performed in order to estimate the partition of active and inactive material in solution and precipitate. Crude insulin hydrochloride (110 mg.) was isoelectrically precipitated by the addition of *N*/100 potassium hydroxide solution (6·5 cc.); the precipitate was collected in the centrifuge, the supernatant fluid

discarded, and the precipitate well stirred with $N/2$ potassium chloride solution. A portion dissolved and was recovered in the usual way from the supernatant fluid obtained after centrifugation. The precipitate was also converted into the picrate and hydrochloride. The undissolved portion weighed 16 mg. and contained 100 units. The solution yielded 21 mg. hydrochloride containing 30 units. The solubility of the isoelectric precipitate in water alone was shown to be negligible under the same conditions by a control experiment.

(2) *Potassium oxalate.* To a solution of insulin hydrochloride (4·00 g. = 34,000 units) in water (50 cc.) a $N/2$ solution of potassium oxalate was run in with stirring. The addition of 10·0 cc. produced the first permanent turbidity. The initial reaction of the solution of hydrochloride, as determined by the hydrogen electrode, was p_H 2·35, and after addition of 24·8 cc. of oxalate this had risen to p_H 3·46. Precipitation was now complete, and precipitate and supernatant fluid were separately converted to picrate and then to hydrochloride. The precipitate after washing and drying weighed 1·14 g. and contained 28,400 units. In this experiment considerable activity was contained also in the supernatant fluid which yielded 1·79 g. hydrochloride containing 3670 units. (In this case the portions insoluble in acid alcohol, during the preparation of the hydrochloride from the picrate, were rejected.)

This experiment shows that oxalate may be used in the same way as potassium chloride for dissolving a considerable portion of material of low activity. Repetition of the process gave a final product which could not be further precipitated by this means without considerable loss. In these later experiments better results were obtained by using more dilute solutions of hydrochloride and of oxalate. Finally, for reasons to be discussed later, $N/8$ potassium oxalate was added to a 1·5 % solution of the hydrochloride. In this way, after four more precipitations, 0·483 g. of hydrochloride was obtained, the unit of which, obtained according to the method laid down in the League of Nations Report [1926], proved to be 0·016 mg., a total recovery of 30,000 units after five precipitations in all. A loss of 10 % on each stage would thus have resulted in a final yield of only 60 %, whereas actually the recovery was almost quantitative. Isoelectric precipitation, carried out by adding $N/10$ sodium hydroxide to the hydrochloride in the usual way, resulted in a loss of approximately 25 % of the activity in each precipitate.

The above example shows that when used for purifying insulin which is already of the order of 0·1 mg. per unit this type of process gives satisfactory purification and yields. Even for this purpose, however, it is troublesome to determine the end-point when complete precipitation is reached, this necessitating repeated centrifugation and addition of further small quantities of oxalate. Moreover, when cruder specimens of insulin are used losses occur. In order to obtain precipitation under uniform conditions the solution was first adjusted by the addition of dilute alkali to p_H 4 ($\pm$ 0·05), experiments having shown that this is an average figure with this material for precipitation in the presence of oxalate. In the following experiment this solution contained 1·0 g.

of crude insulin (= 1250 units) in 25 cc. It was run into 50 cc. of a solution of potassium oxalate and oxalic acid containing five volumes of $N/8$ oxalate to one volume of $N/8$ oxalic acid. The p_H of this solution was 4·05. Although the hydrogen ion concentration remained constant, on running in the insulin solution a precipitate at once began to form. This was collected and converted to hydrochloride, when it weighed 0·264 g. and contained 1200 units. The supernatant fluid, converted to hydrochloride through the picrate, weighed 0·622 g., and the unit was considerably above 2 mg.

(3) *The concentration of oxalate.* These results now made it possible to investigate the optimum concentration of oxalate which should be used, the hydrogen ion concentration remaining constant at p_H 4, in order that a maximum quantity of inactive material might be held in solution, whilst the activity should be found in the precipitate. For this purpose, oxalic acid-potassium oxalate solutions were prepared such that all were at p_H 4 ($\pm$ 0·05), the concentration of oxalate ions being respectively $N/4$, $N/6$, $N/8$ and $N/10$. A solution of crude insulin (3·75 g. = 4300 units) in 250 cc. water was adjusted to p_H 4·02. Equal portions of 60 cc. each were then run into 120 cc. of the above oxalate solutions. After standing for 2 hours, the mixtures were centrifuged, and the precipitates converted into hydrochloride. The results are shown in the following table:

Final dilution of oxalate	p_H after addition of insulin	Wt. of hydrochloride g.	Unit in mg.	Total units recovered %
$N/6$	3·99	0·174	0·17	100
$N/9$	4·00	0·148	0·14	100
$N/12$	3·99	0·113	0·11	100
$N/15$	3·98	0·062	0·15	40

From this it is clear that between $N/12$ and $N/15$ there is an abrupt transition, and that at the lower concentration more than half of the activity remains unprecipitated. The use of higher final concentrations than $N/12$ is to be avoided, however, since a greater proportion of inactive substance accompanies the precipitate, whilst by the use of a concentration of $N/12$ all the activity is carried down with a minimum of other material. The necessity of allowing the mixture to stand for 2 hours in order to ensure complete precipitation should be emphasised.

The effect of concentration of oxalate upon the hydrogen ion concentration at which precipitation of insulin from solution occurred was investigated. The specimen of insulin used had a normal isoelectric point of p_H 5·1.

A solution of insulin hydrochloride (1·00 g.) in water (40 cc.) was treated with $N/10$ sodium hydroxide solution until maximum precipitation occurred (10·2 cc.). The suspension of isoelectric insulin was well shaken and divided among a series of tubes in equal portions. To each of these was added an equal volume of a solution of oxalic acid of double the strength of that finally required. The precipitates dissolved immediately and potassium oxalate of the required normality was run into the clear solutions until precipitation occurred. The

hydrogen ion concentrations were then determined, and the results are shown in Fig. 1.

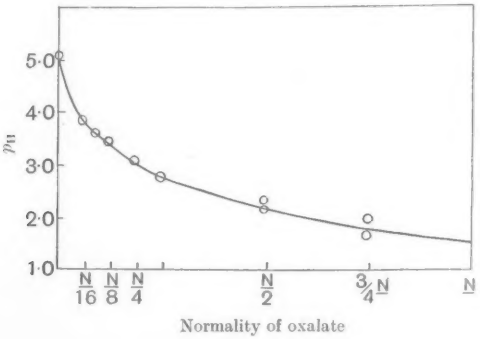


Fig. 1.

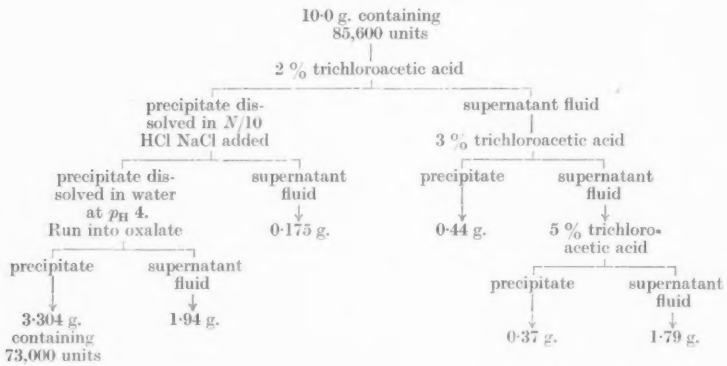
Complete purification process.

The three operations already described were now combined. The following is a typical example. 10·0 g. of insulin containing 85,600 units were dissolved in water and the solution centrifuged to remove a small quantity of insoluble material. The clear solution was diluted to 667 cc. and to this was added with stirring a 10 % solution of trichloroacetic acid (167 cc.). The precipitate settled fairly rapidly, leaving the supernatant fluid only faintly opalescent. After 2 hours the main portion of this was removed by means of a syphon, and the precipitate was collected in the centrifuge. The supernatant fluid gave further precipitates on bringing the concentration of trichloroacetic acid successively up to 3 % and 5 %. These precipitates were collected separately, and these and also the material remaining unprecipitated by 5 % trichloroacetic acid were converted to hydrochloride through the picrate.

The precipitate obtained by 2 % trichloroacetic acid was not converted directly to hydrochloride, but, while still moist, it was dissolved in $N/10$ hydrochloric acid (260 cc.), and to this 130 cc. of saturated sodium chloride solution was run in with good stirring. The precipitate was centrifuged, and the supernatant fluid was neutralised, and, after dilution to avoid precipitation of sodium picrate, the picrate was precipitated. This was converted to hydrochloride also. Meanwhile the salt precipitate was dissolved in water (200 cc.), its reaction was adjusted to p_H 4·0, and the solution was run with good stirring into 400 cc. of the $N/8$ oxalate mixture (p_H 4·05). The precipitate which formed was collected in the centrifuge after 2 hours, the precipitate dissolved in the minimum quantity of dilute hydrochloric acid, the solution diluted to about 200 cc., and, after neutralisation of the excess acid, half its volume of saturated picric acid solution was added. This picrate was well washed in the centrifuge with dilute picric acid solution, and was then converted into the hydrochloride.

Similarly, the material in the supernatant oxalate solution was also converted to picrate and hydrochloride. The weights of hydrochloride obtained at each stage are given in Table II.

Table II.



It sometimes happens that, owing to much salt remaining adherent to the precipitate, on adjusting the hydrogen ion concentration to p_H 4 a precipitate occurs. This may contain a considerable proportion of the activity. It is collected in the centrifuge and redissolved in dilute acid, when the solution may usually be adjusted to p_H 4 without any precipitation occurring. Both the solutions are then run into the oxalate solution separately in the usual way, and the final product is obtained by combining the two precipitates. If the purification by means of oxalate buffer be repeated there will be no precipitate at p_H 4 on adjusting the reaction of the solution prior to oxalate precipitation.

Table III shows the results of a series of experiments where the full procedure was followed.

Table III.

Initial weight g.	Initial unit mg.	Total initial units	No. of oxalate precipitations	Weight recovered g.	Final unit mg.	Total units recovered	Purification ratio
130	6·0	21,600	1	9·0	0·5	18,000	12
136	1·05	129,500	1	15·0	0·125	120,000	8·4
30·5	1·0	30,500	2	2·70	0·09	30,000	11·1
10·0	0·116	85,600	1	3·30	0·045	73,000	2·3
			2	2·53	0·035	72,400	3·3
			5	1·82	0·030	60,600	3·9

Use of sodium citrate. To a solution of insulin hydrochloride (1·0 g. = 1000 units) in 67 cc. water, $N/10$ solution of sodium citrate was added drop by drop. The initial p_H values of the hydrochloride and citrate solutions were 2·4 and 7·3 respectively. After 16 cc. had been added a permanent turbidity appeared, the reaction being p_H 4·5. Complete precipitation was obtained after adding 28 cc. of citrate, when the hydrogen ion concentration had fallen to p_H 5·05.

The precipitate was collected in the centrifuge, and both the precipitate and the material contained in the supernatant fluid were converted into hydrochloride in the usual way. The precipitate yielded 0·254 g. of substance containing 960 units. From the supernatant fluid the weight obtained was 0·52 g., the unit of which was approximately 8 mg. From this experiment it is clear that citrate acts similarly to oxalate in holding in solution a large proportion of inactive material.

Qualitative tests.

The following tests were performed on specimens of insulin the rabbit units of which lay between 0·016 and 0·03 mg. Solutions containing 2 mg. or more per cc. were used. The Pauli reaction was strongly positive with all the specimens tested, whether in the usual way or after destruction of the tyrosine by nitration by Brunswick's method [cf. Shonle and Waldo, 1924]. The biuret, Millon and xanthoproteic reactions were also always positive. The reaction for tryptophan and the Molisch reaction were negative. If carefully neutralised before performing the test, all specimens gave a positive ninhydrin reaction. Jaffé's reaction with alkaline picric acid was positive, which may indicate, according to Abderhalden, the presence of diketopiperazine rings. Although these experiments were performed quantitatively whenever possible, in no case was the intensity of the reaction proportional to the activity of the preparations used.

Heat coagulation. A solution of purified insulin hydrochloride in 1·5 % aqueous solution is not coagulated if the temperature is slowly raised to 100°. When a suspension of the isoelectric precipitate is heated in the same way it coagulates, the activity being contained almost completely in the precipitate. The coagulated precipitate so obtained may be dissolved in dilute acid, showing that this is not an irreversible coagulation. The precipitate obtained on adding sodium chloride solution to a solution of insulin hydrochloride, when treated similarly, is irreversibly coagulated.

Dialysis. A solution of insulin hydrochloride (0·500 g. containing 2000 units) previously purified by precipitation with 2 % trichloroacetic acid, as described above, was dialysed in a collodion thimble against $N/10$ hydrochloric acid for 18 hours. At the end of this time the level of liquid inside the thimble was higher than that outside, and the outer liquid had taken on a yellowish tinge. Both the inside and outside fluids were neutralised, and the dissolved material was precipitated as picrate. It was found that 0·147 g., almost a third of the total weight of substance, had passed through the membrane, and when tested upon rabbits this was found to contain approximately 15 % of the total initial activity. A similar result is reported by Shonle and Waldo [1924]. The dialysate is completely precipitated by 2 % trichloroacetic acid and there was only a slight difference in the ratio of amino- to total nitrogen, determined first on the original material and secondly on the dialysate, as shown in Table IV. No difference was observed in the unit of the dialysate and dialysed solution, when material which had been previously precipitated

Table IV. *Dialysis.*

	Weight in g.	Amino-N %	Total N %	Amino-N / Total N %
Inside	0·259	0·845	14·4	5·85
Outside	0·147	0·98	14·2	6·9
Original material	0·500	0·817	14·1	5·8

with 2 % trichloroacetic acid was employed. After prolonged dialysis (5 days) the whole of the material was found to pass through a similar membrane, the outer liquid being replaced by fresh distilled water at 24-hourly intervals.

None of the material passed through a parchment membrane (Schleicher and Schüll) when treated in an exactly similar way.

Precipitation by tungstic acid. A solution of insulin hydrochloride was precipitated by tungstic acid, as in the Folin method of blood analysis, the whole of the activity being carried down.

Effect of heat on the trichloroacetic acid precipitate. It was observed that on warming a suspension in 2 % trichloroacetic acid of the trichloroacetic acid precipitate, which had previously been washed in the centrifuge with 2 % trichloroacetic acid, the suspension cleared slightly. The solution was warmed to 60° and was filtered through analytical filter paper in a jacketed funnel maintained at 60°. The clear filtrate, on cooling, became turbid. This was centrifuged, and the precipitate was converted to hydrochloride in the usual way. This precipitate contained 6 % of the weight of the original specimen and approximately 6 % of the initial activity. Experiments on this point are being continued. Similar results were obtained on heating a suspension of the precipitate, caused by the addition of sulphosalicylic acid to insulin solutions.

An attempt was made to prepare from our highly purified specimens insulin in a crystalline state, following the method of Abel [1926], but without success.

Quantitative determinations.

Inorganic constituents. By the process described it is possible to obtain material which leaves a very slight ash on incineration. 4·663 mg. (unit = 0·03 mg.) yielded 0·004 mg. ash.

The *sulphur* content of insulin preparations of varying degrees of activity was determined by Carius' method. The results are shown in Table V.

Table V.

Unit (mg.)	1·5	1·5	1·0	0·3	0·25	0·2	0·15	0·06	0·03
Sulphur %	1·7	2·0	1·9	1·6	1·5	1·9	2·0	2·0	1·55

The sulphur content shows but slight variation even in specimens of insulin differing in activity in the ratio of 50 to 1.

Nitrogen. The amino-nitrogen and total nitrogen content of insulin hydrochloride has been determined in a number of cases. The values obtained vary only slightly for specimens of very different degrees of purity, the units of specimens investigated lying between 0·15 mg. and 0·016 mg. The total nitrogen

varied from 13·7 to 15·0 %, the ratio of amino-nitrogen to total nitrogen from 5·33 to 6·9 %. All these preparations had previously been precipitated by 2 % trichloroacetic acid. These results are similar to those obtained by Scott [1925]. We have also analysed by van Slyke's method a specimen of insulin the unit of which was 0·03 mg. We have added for comparison the figures obtained by us on a specimen of very crude insulin hydrochloride containing 1·5 mg. of solids per unit. The results are given in the following table:

Unit	1·5 mg.		0·03 mg.	
Ammonia-N	9·43		9·59	
Melanin-N	1·29		0·52	
Cystine-N	6·06		5·93	
Arginine-N	15·28	29·28	9·37	19·8
Histidine-N	2·93		4·29	
Lysine-N	5·01		0·21	
Amino-N (non-basic)	52·64	63·12	67·61	72·75
Non-amino-N (non-basic)	10·48		5·14	
Total N (sum)	103·1		102·6	

Apart from a marked difference in the cystine nitrogen and a reduction in the lysine content in our purified material, there is no very striking difference between the figures obtained by Scott and by us, although the material analysed by us was approximately four times purer than his. These results show the danger of speculating on the "purity" of insulin preparations on account of similarity between such analyses; see, for example, the conclusions of Somogyi, Doisy and Shaffer [1924].

Summary.

1. Insulin is completely precipitated with the protein and metaprotein class, following the analytical method of Wasteneys and Borsook.

2. On this basis a method of purification has been developed, depending on the action of salt solutions at the isoelectric point, which can be used to produce insulin of a very low physiological unit (about 0·02 mg.).

3. The results of various qualitative tests on the purified product are described.

4. Analytical results on specimens of insulin of varying degrees of purity are given.

5. The bearing of these observations on the probable nature and constitution of insulin is discussed.

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LXXX. A NOTE ON THE WEIGHT OF THE RAT DURING GESTATION.

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Many observers have demonstrated the now well-established fact that a nursing mother can sacrifice her own tissues for the benefit of her suckling young, and it has been shown that a rat can lose as much as one-third of her weight during the lactation period [Hartwell, 1922]. The loss in weight of the mother is correlated with a poorness in *quality* of her diet, and a problem which at once presents itself is: does a similar sacrifice of the maternal organism occur during gestation?

The earlier research work on pregnancy was mainly along metabolic lines and was based on an analysis of excreta, the N balance being the primary consideration, while the weight of the animal was rarely recorded. A summary of this earlier work is given by Marshall [1922] who points out that the majority of the experiments were made before the vitamins were known and therefore are of little value now. According to the same writer, Cramer finds that rats fed on a diet of bread, rice and maize maintained their weight at the end of pregnancy, but when the diet was supplemented by yeast preparations, the mother always showed a considerable gain in weight during this period.

Feldman [1920], summing up the chief points of Bar's researches, regards pregnancy as a "state of symbiosis" and considers that there should be no loss to the maternal organism, provided the diet is satisfactory, but if it is not, then the results are disastrous, both for the mother and her foetus. In some earlier experiments [Hartwell, 1924, 2] when the rats were receiving a notably poor diet, it was found that there was little or no loss in the mother's weight at the end of gestation, so it was thought advisable to work out any available data in this respect.

Experimental.

(*a*) *Methods.*

The rats from which the data given in this paper have been collected are piebald animals bred in the laboratory. In most of the experiments 6 ♀ and 6 ♂ were used and were kept in a cage together; the method of weighing, feeding, etc., has been fully described in previous papers [Hartwell, 1926 and earlier]. The number of young in each litter was reduced to six, and the doe was returned to the bucks at the end of the suckling period (21 days); she

was not allowed a "rest" as given by other workers [Smith and Chick, 1926]. According to Donaldson [1924] and Greenman and Duhring [1923] the gestation period is 22 days, but 23 days was taken in these experiments, because it has been found that litters are never born before 23 days after mating. The difference in weight at the beginning and end of gestation given in the table was obtained as follows: the mother rat was weighed the day after the litter was born and the beginning of pregnancy was taken as approximately 23 days before the birth of the young.

(*b*) *Diets.*

The proportions used in the different foods were:

I *a*. 30 g. white bread, 3 g. butter, 0·7 g. salt mixture, water [Hartwell, 1924, 1 and 2].
I *b*. 30 g. brown bread, 3 g. butter, 0·7 g. salt mixture, water [Hartwell, 1924, 2].
II. 30 g. white bread, 12 g. food casein, 3 g. butter, 0·7 g. salt mixture, water [Hartwell, 1924, 3].
III *a*. 30 g. white bread, 3 g. food casein, 3 g. butter, 0·7 g. salt mixture, water [Hartwell, 1924, 3].
III *b*. 30 g. white bread, 3 g. ash-free casein, 3 g. butter, 0·7 g. salt mixture, water.
IV. 30 g. white bread, 3 g. gluten, 3 g. butter, 0·7 g. salt mixture, water [Hartwell, 1924, 3].
V. 100 g. oatmeal, 2·88 g. salt mixture, 14 g. butter, 500 cc. water [Hartwell, 1926].
VI. 100 g. boiled potato, 5 g. butter, 1 g. salt mixture, 25 cc. water [Hartwell, 1927].
VII. 15 g. white bread, 4·3 g. dried milk, 36 cc. water [Hartwell, 1925, 2].
VIII. 15 g. white bread, 36 cc. cow's milk [Hartwell, 1925, 2].
IX. 15 g. white bread, 36 cc. diluted evaporated milk [Hartwell, 1925, 2].
X *a*. Synthetic diet [Hartwell, 1925, 1].
X *b*. The same as X *a*, but containing more marmite.
XI. Controls fed on kitchen scraps supplemented by bread and milk.

Results and comments.

A striking feature is the almost constant gain in weight of the mother during gestation. The actual differences in weight are summed up in the following table.

			Weight of does at beginning and end of gestation								
						Average		Gain		Loss	
Diet	Number of rats	Number of litters born	Number of times weight gained	Number of times no loss or gain	Number of times weight lost	gain g.	loss g.	max. g.	min. g.	max. g.	min. g.
I *a* (*Exp.* 1)	3	9	9	0	0	18	0	37	9	0	0
(*Exp.* 2)	6	11	11	0	0	20	0	35	5	0	0
(*Exp.* 3)	6	12	12	0	0	23	0	37	14	0	0
I *b*	5	13	13	0	0	25	0	42	7	0	0
II	6	17	14	1	2	19	8·5	36	1	17	10
III *a* (*Exp.* 1)	6	35	30	1	4	21	20	58	1	39	10
(*Exp.* 2)	6	34	31	1	2	20	6	47	1	7	5
III *b*	6	12	12	0	0	26	0	47	13	0	0
IV	6	17	11	0	6	19	8	38	1	15	3
V	5	14	12	2	0	20	0	55	5	0	0
VI	3	7	4	0	3	11	10	22	3	20	5
VII	6	15	12	0	3	20	10	48	1	14	4
VIII	6	15	14	0	1	24	20	43	4	20	20
IX	6	20	20	0	0	22	0	44	1	0	0
X *a* (*Exp.* 1)	6	34	29	0	5	18	7	45	2	18	1
(*Exp.* 2)	4	21	17	1	3	18	10	40	5	14	7
X *b* (*Exp.* 1)	6	35	35	0	0	27	0	57	4	0	0
(*Exp.* 2)	9	28	27	1	0	26	0	58	2	0	0
(*Exp.* 3)	7	14	14	0	0	30	0	66	14	0	0
XI	6	40	37	0	3	26	5	68	3	10	1
Totals	114	403	364	7	32						

The diets used were very varied, and one or two were extremely poor. In order to make some sort of comparison the following table was drawn up to show the approximate amounts of fat, protein, carbohydrate and mineral salts in each diet. The figures are based on analyses given by Plimmer [1921].

Diet	Protein	Fat	Carbohydrate	Mineral salts
I *a*	11	12	71	6
II	39	8	46·5	6·5
III *a*	20	11	63	6
III *b*	21	11	63	5
IV	20	12	63	5
V	11	19	·66	4
VI	8	17	66	8
VII	16·5	8·5	71	4
VIII	17	9·5	69·5	4
X	20	12	64	4

The carbohydrate and mineral salts contents of all the diets are probably within normal limits; the fat shows a wide range, but in all cases sufficient butter was given to ensure an adequate supply of vitamins A and D. Incidentally there must have been the necessary ration of vitamin E, otherwise reproduction would not have occurred. The protein of the different diets is the factor with the greatest variation, both in amount and quality. Diet VI (potato) is obviously low in protein, while diet II, with 39 %, contains an excess, since diet III *a* provides for practically maximum growth with only 20 % of the same proteins. Although diets III *a* and IV contain equal amounts of protein, the former must be regarded as a better food because it has three proteins instead of one, the protein of bread being supplemented by the proteins of milk, while in diet IV the added protein is gluten.

Taking as a criterion the rate of growth of young rats on these foods, diets VI, I *a* and I *b* are by far the worst, yet with I *a* and I *b* there was never any loss of weight during gestation. No stress can be laid on the number of litters born, because this depended entirely on the duration of the experiment. The number per litter and the weight of the young at birth varied considerably, but in no case did the experimental animals come up to the controls in either respect; moreover, with the poorer diets many of the young were born dead. It might be expected that the rat would show the greatest gain with her first pregnancy, since she breeds young and when by no means fully grown. The gain in weight at the first gestation was usually, but by no means always, large, and the gain did not decrease with each successive pregnancy. The gain in weight of the mother rat bears no relation to the size of the litter, so the eating of the placentae cannot be put forward as an explanation of the increased weight. It appears, therefore, that the characteristic sacrificing of the mother for her offspring, a noticeable feature of the lactation period, does not occur during gestation. The low weight of the young at birth, and the fact that many are frequently born dead when the mother is given a poor diet, may also be regarded as evidence in favour of this statement.

SUMMARY.

1. Out of 403 litters born, in 364 cases the mother gained weight during gestation.

2. This gain in weight appears to be of constant occurrence and to take place when the mother is fed on a poor as well as on a good diet.

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LXXXI. A STUDY OF THE SYSTEM CARBONIC ACID, CARBON DIOXIDE AND WATER.

I. DETERMINATION OF THE TRUE DISSOCIATION-CONSTANT OF CARBONIC ACID.

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Although the quantitative equilibrium relations in the bicarbonate-carbonic acid systems are very accurately known in biological solutions, it is a remarkable fact that only little is known about the way in which this equilibrium is attained and about the velocities of the reactions occurring. Still it might be supposed that this equilibrium is not reached at once, as in purely ionic reactions, but that it is a very definite time-process under certain conditions. Thus the buffer-capacity of a bicarbonate system, after addition of a stronger acid, is calculated from the apparent dissociation-constant ($k_1 = 3 \times 10^{-7}$), but it is possible that initially the system is reacting according to the true constant of carbonic acid ($k_1 = 4 \times 10^{-4}$) during a biologically significant time, with a consequent large temporary increase of the resulting c_H. If the carbonic acid were produced continuously, it might then be acting continuously as a strong acid.

On the other hand, if we consider the hydration of CO_2 as a relatively slow time-reaction, the transport of this substance by the blood must be greatly influenced by its velocity of hydration, as was pointed out by Collingwood [1924].

Many questions present themselves in this way. What is the velocity of the hydration of carbonic acid *in vivo*; can H_2CO_3 exist there as a strong acid for a sufficiently long time? How is the velocity of dehydration influenced by temperature, $[H^+]$, $[HCO_3^-]$, other ions, CO_2 tension, etc.? We will try first to investigate the velocity of dehydration of carbonic acid in a pure bicarbonate solution, and to estimate its true dissociation-constant. Then we will study these properties in a more physiological solution and in the circulating blood.

The hydration of CO_2 and dehydration of H_2CO_3.

Since the work of McBain [1912], Thiel [1913, 1916, 1922], Strohecker [1916], Thiel and Strohecker [1914, 1916], Pusch [1916] and especially of Faurholt [1922, 1924], it has been recognised that a solution of CO_2 in water

contains only a small amount of the rather strong acid, H_2CO_3. If base is added to such a solution only a fraction, equivalent to the amount of H_2CO_3 present, is neutralised at once; the remaining large fraction is neutralised as a time-reaction with practically the velocity of the reaction $CO_2 + OH^- \rightarrow HCO_3^-$. Calculation of the true dissociation-constant from the fraction of pre-existing H_2CO_3 by Thiel and Strohecker, gave an approximate value of $K_{H_2CO_3} = 4{\cdot}4 \times 10^{-4}$ (in a 0·008 mol. solution of carbonic acid at 4°, 1·23 % is present as H_2CO_3). The velocity of this hydration, as studied by Thiel and Strohecker, appeared to be dependent upon the p_H and to increase with increasing alkalinity.

Results of more detailed character have been obtained by Faurholt. His method makes use of the reaction between CO_2 and amines (the carbamino-reaction), so that in a mixture of $CO_2 + H_2CO_3$ the HCO_3^- can be precipitated as $BaCO_3$, while the CO_2 is prevented from precipitation by addition of ammonia, methyl- or dimethyl-amine. Faurholt's conclusions are the following:

Hydration of carbon dioxide is occurring in two ways, viz.:

$$CO_2 + H_2O \rightarrow H_2CO_3, \quad \frac{d[CO_2]}{dt} = -k_1[CO_2]$$

and

$$CO_2 + OH^- \rightarrow HCO_3^-, \quad \frac{d[CO_2]}{dt} = -k_2[OH^-][CO_2],$$

k_1 at 0° = 0·0013 and at 18° = 0·0011, k_2 at 0° = $10^{42{\cdot}61}$ and at 18° = $10^{43{\cdot}24}$.

Both processes are occurring simultaneously, so the hydration is determined by

$$\frac{d[CO_2]}{dt} = -(k_1 + k_2[OH^-])[CO_2],$$

which indicates that the reaction is of monomolecular type if $[OH^-]$ is constant. If $p_H < 8$, $[OH^-]$ is so small that the reaction has practically the type $CO_2 + H_2O \rightarrow H_2CO_3$; if $p_H > 10$ the second reaction $CO_2 + OH^- = HCO_3^-$ is predominant. In the range p_H 8–10 the velocities of both reactions are important.

The dehydration of carbonic acid is taking place in two ways:

$$H_2CO_3 \rightarrow H_2O + CO_2 \quad \frac{d[H_2CO_3]}{dt} = -k_3[H_2CO_3],$$

$$HCO_3^- \rightarrow CO_2 + OH^- \quad \frac{d[HCO_3^-]}{dt} = -k_4[HCO_3^-],$$

k_3 at 0° = ± 1, at 18° = 7·1, k_4 at 0° = $10^{-5{\cdot}52}$, at 18° = $10^{-4{\cdot}52}$.

Thus the dehydration of H_2CO_3 is a monomolecular process; Faurholt deduces that the dehydration of the bicarbonate is also monomolecular if the p_H is constant. Between p_H 8 and 10 both processes have velocities of similar magnitude, in more acid solutions $H_2CO_3 \rightarrow H_2O + CO_2$ predominates, in alkaline solutions the dehydration of HCO_3^- prevails. In acid fluids dehydration is practically complete; between p_H 5 and 8 CO_2 and carbonic acid can be present; in alkaline ranges hydration is nearly complete.

The time for 90 % establishment of equilibrium in strongly acid solutions is nearly independent of p_H; at 0° this time is 1 sec., at 18° ½ sec. At $p_H = \pm 8$, however, this time is maximal, at 0° about 700 sec., at 18° about 80 sec.; from p_H 9 the time decreases regularly. These times have been calculated for infinite dilution and must be modified for the presence of electrolyte concentrations. Faurholt gives a probable error of 50 %.

For equilibrium conditions he finds:

$[H_2CO_3] \rightarrow k_{\text{hydration}}[CO_2]$, $k_{\text{hydration}} = \frac{1}{300}$.

$[H^+] \times [HCO_3^-] \rightarrow k_{H_2CO_3} \cdot [H_2CO_3]$, $k_{H_2CO_3}$ at 0° = ± 2 × 10^{-4}.

$[H^+] \times [CO_3^=] \rightarrow k_{HCO_3^-} \cdot [HCO_3^-]$, $k_{HCO_3^-} = 10^{-10{\cdot}5}$ (0°), $10^{-10{\cdot}3}$ (18°).

Although the value of the data given by Faurholt is certainly important for biological reactions, many of them are only approximate; especially in the physiological range of p_H and temperature many questions remain unsolved. In order to make an accurate estimation of the important true dissociation-constant of carbonic acid and the types and velocities of its dehydration under physiological conditions, we have directly recorded with a lag of $< \frac{3}{5}$ sec. the changes of p_H with an accuracy $> 0{\cdot}005$ p_H of a bicarbonate system, after quickly mixing it with acid.

Method.

It will be clear that a method intended not only to measure but also to record p_H continuously cannot be a compensation method with zero instrument, but must be suited to record the potential differences between the unknown electrodes and constant electrodes without any appreciable polarisation. Therefore we adapted a method similar to that of Goode [1922] and followed the changes of p_H accurately and with a time lag as short as possible by connecting a suitable type of hydrogen electrode system, compensated by a potentiometer circuit, with the grid of a common triode valve and recorded the changes of the (compensated) plate current with a suitable recording galvanometer. The method is illustrated by the diagram of Fig. 1.

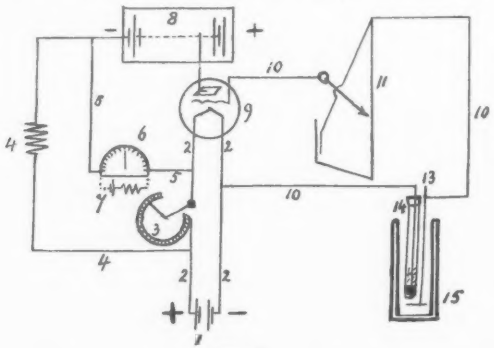

Fig. 1.

The filament of a Philips B 406 valve (9) is heated by a 4 v. accumulator (1) through the circuit (2) and the resistance (3). The plate current flows through the anode battery (8), the circuit (5) and the galvanometer (6), and can be compensated by the shunt with resistance (4). In this scheme the compensating potential is taken from the heating potential, so that a large plate potential will take too much energy from the heating cells for compensation. Therefore the maximum plate potential in this scheme can only be 30 v., and the sensitivity of the galvanometer was about 4 mm. for 1 mv. tension on the grid. This sensitivity may be increased about tenfold by removing the shunt (4), bringing the plate potential to 80 v. and compensating the plate current by a separate 2 v. accumulator, with resistance (7). In such a system it is sometimes necessary to shunt a large capacity condenser across the anode battery.

The E.M.F. of the measured system is connected between the grid and the negative pole of the heating battery (circuit 10), and can be compensated and measured by the potentiometer (11).

As experimental electrodes we used the quinhydrone system with bright Pt electrodes, the reaction time of which is as short as possible. As second electrode we used a saturated calomel electrode, or an Ag—AgCl—KCl electrode, or a narrow tube with phosphate solution (equal parts of 1/15*N* KH_2PO_4 and Na_2HPO_4, $2H_2O$), quinhydrone and a long bright Pt wire which reaches to the cotton plug by which the bicarbonate and phosphate solutions are separated. This second electrode may be cemented to the moving electrode to ensure constant distance between the two, but this proved to be unnecessary. It is important that the resistance of the electrodes is small, in order to prevent the influence of induction potentials on the measured E.M.F. If this precaution is taken and if all parts of the apparatus which can come into contact with the grid circuit have been carefully insulated, the recording of the E.M.F. of the system under study is absolutely undisturbed.

An essential part of this research was the completeness of mixing of the reacting fluids in as short a time as possible. The following method was found to be the best.

The $NaHCO_3$ solution (100 cc. in a Dewar flask, diameter 3 cc., height 14 cm.) is stirred continuously by the Pt electrode itself which is a disc of 2 cm. diameter and moves up and down in the solution over a distance of 6 cm. twenty times a second. The connecting parts of this electrode are insulated with De Khotinsky cement, so that only the disc remains uncovered. The moving electrode is connected to the potentiometer circuit by very flexible wire and all contacts are soldered. The diluted HCl (0·95 cc.) is injected into the bicarbonate solution by means of an automatic 1 cc. syringe with strong springs; attached to the nozzle of the syringe is a straight glass tube of 0·3 cm. diameter which reaches half-way down the Dewar vessel. Through this tube the acid is injected automatically in less than 0·1 sec.

The variation in distance between the moving Pt electrode and the second constant electrode causes no deflection of the galvanometer if the resistance of these electrodes is not too high.

It might be supposed that the change of E.M.F. which follows upon the injection of HCl into the bicarbonate solution is initially caused by a temporary local concentration of free HCl at the electrode. If, however, the velocity of the mixing electrode is high enough (> ten movements per sec.) this possibility seems excluded by the following experiments:

(*a*) If the same amount of 0·01*N* HCl is injected into 100 cc. of another equimolar buffer solution (acetate-phosphate) a temporary rise of c_H is not noticed, but the E.M.F. is raised to its end-point in a time which is no greater than the lag of the recording system ($< \frac{3}{25}$ sec.) (mixing time and reaction time of the quinhydrone electrode).

(*b*) The temporary rise in the curve indicating the c_H of the bicarbonate solution (H_2CO_3 peak) does not occur in any HCO_3'—H_2CO_3 system in which

HCl is injected, but only in those systems, in which the p_H is less than 7·5[1]. If this peak were caused by free HCl, we can find no reason why it should present itself only on the acid side of p_H 7·5. Moreover, if one causes such a peak by injecting HCl on a slowly moving electrode, the decrease of the curve is as rapid as the increase, while the decrease of the H_2CO_3 peak is much more gradual and corresponds exactly to the deduced velocity of dehydration.

Determination of the true dissociation-constant of H_2CO_3.

In order to measure the true dissociation-constant of H_2CO_3 we recorded the p_H of 100 cc. 0·02*N* $NaHCO_3$, saturated with CO_2, into which 0·95 cc. 0·01*N* HCl was quickly injected and mixed (see fig. 2 and Table I). The formation of H_2CO_3 causes here a rapid rise of $[H^+]$, which is followed at once by a slower decrease, owing to the dehydration process $H_2CO_3 \rightarrow H_2O + CO_2$. Our object now was to extrapolate from this registered curve of E.M.F. the initial c_H of the solution, as if no dehydration had occurred.

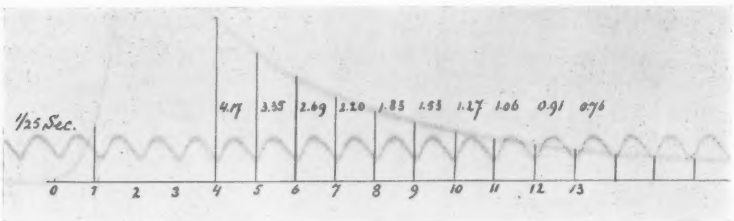

Fig. 2.

To do this, we may suppose the dehydration to be a monomolecular process at this reaction (p_H 5–6), which finally reaches a dynamic equilibrium with the inverse hydration of CO_2.

Then the velocity of this reaction would be described by

$$-\frac{d[H_2CO_3]}{dt} = k_1[H_2CO_3] - k_2[CO_2];$$

but

$$[H_2CO_3] = \frac{[HCO_3^-].[H^+]}{K}$$

(K = the true dissociation-constant of H_2CO_3),

therefore

$$-\frac{[HCO_3^-]}{K} \cdot \frac{d[H^+]}{dt} = k_1 \frac{[HCO_3^-]}{K} \cdot [H^+] - k_2[CO_2].$$

$[HCO_3^-]$ is practically constant, as the small change in its concentration caused by the dehydration of H_2CO_3 may be neglected in regard to the total amount of $NaHCO_3$ in solution. $[CO_2]$ is also a constant as the solution is continuously kept saturated with the gas. So we have:

$$-\frac{d[H^+]}{dt} = k_1 \cdot [H^+] - k_2',$$

or by integration $[H^+] = C \cdot e^{-k_1 t} + D$, in which C and D are new constants; where D may be determined by the c_H at equilibrium, *i.e.* after infinite time.

[1] The influence of the reaction on the type of buffering of a bicarbonate solution will be described in the next paper of this series.

In this way we get $[H^+] = C \,.\, e^{-k_1 t} + [H^+]_\infty$, or

$$\log ([H^+] - [H^+]_\infty) = - k_1' t + \log C \quad (k_1' = 0{\cdot}434 \times k_1).$$

The experimental values of $\log ([H^+] - [H^+]_\infty)$ should be a linear function of the time. All curves calculated in this way showed a convexity towards the plane axis, indicating that k_1 is not a constant but decreases during the reaction, which consequently cannot be monomolecular.

It was now natural to suppose that the hydrogen ions have a catalytic influence on this dehydration, as they have in many hydrations and dehydrations (formations of lactones and the inverse reactions, inversion of sucrose, etc.). Pusch [1916] made the same assumption for the hydration of CO_2.

In this case the equation becomes

$$- \frac{d\,[H_2CO_3]}{dt} = k_1\,[H_2CO_3]\,[H^+] - k_2\,[CO_2]\,.\,[H^+];$$

taking $$[H_2CO_3] = \frac{[HCO_3^-].[H^+]}{K}$$

(K = the true dissociation-constant of H_2CO_3),

we get $$- \frac{d\,[H^+]}{dt} = k_1\,[H^+]^2 - k_2\,.\,[CO_2]\,.\,\frac{K}{[HCO_3^-]}\,.\,[H^+].$$

Let $$k_2\,.\,[CO_2]\,.\,\frac{K}{[HCO_3^-]} = k_3,$$

then $$- \frac{d\,[H^+]}{dt} = k_1\,[H^+]^2 - k_3\,[H^+].$$

By integration $ln\,\frac{k_1\,[H^+] - k_3}{[H^+]} = - k_3 t + C$ in which C is an integration constant.

If $\frac{k_1}{k_3} = \alpha$ and $\frac{1}{[H^+]} = p$ then $ln\,(\alpha - p) = - k_3 t + C$. ...(A)

If α is known, $\log\,(\alpha - p)$ may be plotted as a function of time. α was calculated in the following way.

Substituting the determined values of p for equal intervals of time, then

$$\frac{\alpha - p_n}{\alpha - p_{n+1}} = \frac{\alpha - p_{n+1}}{\alpha - p_{n+2}} = \ldots\ldots = e^{-k_3(t_n - t_{n+1})} = \beta,$$

as $$t_n - t_{n+1} = t_{n+1} - t_{n+2} = \ldots.$$

Thus $$\alpha - p_n = \beta\,(\alpha - p_{n+1}) \text{ or } p_n = \beta p_{n+1} + \alpha\,(1 - \beta).$$

By taking p_n as a function of p_{n+1}, the formula must represent a straight line; β in this formula is the tangent of the angle with a plane axis of the constructed p_n/p_{n+1} line, which actually appears to be a straight line (Fig. 3). Thus β can be determined graphically; $\alpha\,(1 - \beta)$ is the portion which is cut from the vertical zero line. From the graph can be seen the portion which is cut from the plane axis of $p_{n+1} = 5{\cdot}5$; from this portion $\alpha\,(1 - \beta)$ can be determined, and as β is known, α may be calculated.

This method of calculating α has the advantage that all observations on the experimental p_H curve may be used; this would not have been possible if α had been calculated from the limit-condition of the integral. The calculation of α in this way and its substitution in equation (A) gives in fact a very good straight line (Fig. 4). This forms a strong argument for our supposition that hydration and dehydration of carbonic acid are proportional to the $[H^+]$ of the system.

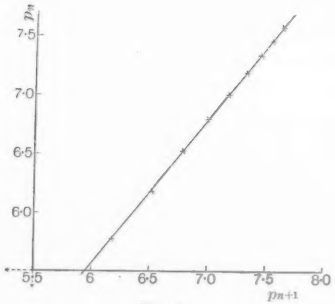

Fig. 3.

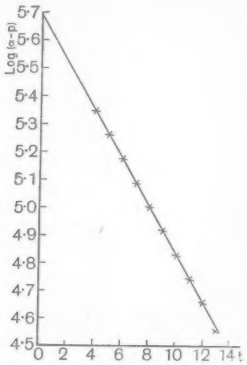

Fig 4.

We are now able to extrapolate the $[H^+]$ of the bicarbonate solution immediately after injection of the HCl. As an example we take the curve reproduced in Fig. 2 which had been obtained by injecting 0·95 cc. of 0·01*N* HCl into 100 cc. 0·02*N* $NaHCO_3$ at 14°. The values calculated from this curve are combined in Table I.

Table I.

t.	cm.	Δ mv.	p_H*	$p = \left(\frac{1}{[H^+]}\right).10^5$	$(a - p).10^5$	$\log (a - p)$
4	4·17	8·45	5·762	5·78	2·22	5·346
5	3·35	6·79	5·791	6·18	1·82	5·260
6	2·69	5·45	5·814	6·52	1·48	5·170
7	2·20	4·46	5·832	6·79	1·21	5·083
8	1·83	3·71	5·845	7·00	1·00	5·000
9	1·53	3·10	5·856	7·18	0·82	4·914
10	1·27	2·57	5·865	7·33	0·67	4·826
11	1·06	2·15	5·872	7·45	0·55	4·740
12	0·91	1·84	5·878	7·55	0·45	4·653
13	0·76	1·54	5·883	7·64	0·36	4·556

Calibration of the curve 10 mv. = 4·94 cm.
Initial p_H before addition of HCl = 5·91.
Graphical determination of $\beta = 1{\cdot}221$.
Graphical determination of $a = 8{\cdot}00 \times 10^5$.
$k_2 = 2{\cdot}16$ (Brigg. log., time in sec.)†.
So $k_1 = a \times k_2 = 8{\cdot}00 \times 10^5 \times 2{\cdot}16 = 1{\cdot}73 \times 10^6$.
Found by extrapolation $\log (a - p_0) = 5{\cdot}68$ (see Fig. 4).
$p_0 = 3{\cdot}16 \times 10^5$.
$H_0 = 3{\cdot}16 \times 10^{-6}$.

* The absolute strength of these p_H values has not been determined in three decimals, but the relative differences in p_H, which are the only important values for the determination of a and β, may be given in three decimals.

† k_2 is, according to equation (A), equal to the tangent of the angle which the straight line of Fig. 4 makes with the plane axis.

The true dissociation-constant K was calculated as follows.

Concentration of H before injection of HCl $= H_1$
,, ,, H immediately after the injection $= H_0$
,, ,, H_2CO_3 before injection $= c_1$
,, ,, HCO_3^- before injection $= c_2$
,, ,, HCl (diluted to 100 cc.) $= c_3$

After the injection of HCl the concentration of H_2CO_3 must be $c_1 + c_3$ and of $HCO_3^- = c_2 - c_3$.

Before injection we have $c_1 \times K = H_1 \times c_2$,

and after $(c_1 + c_3) \times K = H_0 \times (c_2 - c_3)$,

so $$K = \frac{H_0 \times (c_2 - c_3) - H_1 \times c_2}{c_3}.$$

In our example we had $H_1 = 1{\cdot}23 \times 10^{-6}$, $H_0 = 3{\cdot}16 \times 10^{-6}$, $c_2 = 2 \times 10^{-2}$, $c_3 = 9{\cdot}5 \times 10^{-5}$, so K at $14° = 4{\cdot}0 \times 10^{-4}$.

We have made twelve similar experiments at temperatures between 14° and 18° and always found values between $K = 1{\cdot}5$ and 4×10^{-4}. Researches in which the constancy of temperature is considered, and determinations at 37° are in progress.

The graphical determination of β gave a k_3 of 2·16; as $\alpha = \frac{k_1}{k_3}$ we find

$$k_1 = 8{\cdot}00 \times 10^{-5} \times 2{\cdot}16 = 1{\cdot}73 \times 10^{-6}.$$

Thus at $p_H = 6$, the dehydration constant of $H_2CO_3 = 1{\cdot}73$, which indicates that the time for semi-completion of the dehydration is 0·17 sec. at 14°.

If it is permissible to extrapolate this value for a physiological p_H of 7·35, we get for semi-completion about 4 sec. So it seems quite probable that the existence of H_2CO_3 in a bicarbonate solution may occur for a biologically important time.

Hydration of CO_2 at p_H 7 seems to be a very slow reaction (cf. Collingwood [1924]).

The relation of the constants of hydration and dehydration is given by the equilibrium constant of $CO_2 + H_2O \rightleftarrows H_2CO_3$:

$$\frac{k_{\text{hydration}}}{k_{\text{dehydration}}} = K_{\text{equilibrium}} = \frac{[H_2CO_3]}{[CO_2]} = \text{(approx.)}\ \frac{\text{apparent diss.-const. of carbonic acid}}{\text{true diss.-const. of carbonic acid}}$$

$$= \frac{3{\cdot}10^{-7}}{4{\cdot}10^{-4}} = 7{\cdot}5 \times 10^{-4}.$$

Another way of calculating the equilibrium constant is the following. According to Strohecker there is in a certain solution of carbon dioxide 99·44 % present in the form of CO_2 and 0·05 % as undissociated H_2CO_3; this gives for the equilibrium constant $\frac{0{\cdot}05}{99{\cdot}44} = 5 \times 10^{-4}$, which agrees fairly well with the calculation from the true and apparent dissociation-constant, but not with Faurholt's value. If we calculate the hydration constant of CO_2 from the relation

$$\text{hydration constant} = 7{\cdot}5 \times 10^{-4} \times \text{dehydration constant},$$

we get at p_H 7·35

$$k_{\text{hydration}} = 7{\cdot}5 \times 10^{-4} \,.\, k_{\text{dehydration}} = 5{\cdot}85 \times 10^{-5},$$

and the time for semi-completion of hydration is $\frac{0{\cdot}301}{5{\cdot}85 \times 10^{-5}} = 5{\cdot}2 \times 10^3$ sec. or about $1\frac{1}{2}$ hours. Moreover, the inverse reaction is retarding hydration, but it might be possible that the reaction $CO_2 + OH^- \rightarrow HCO_3^-$ is coming into play at this p_H.

The true dissociation-constant of H_2CO_3 being 4×10^{-4}, we may now estimate the buffer value of the bicarbonate solution, immediately after the addition of the acid.

Van Slyke's formula for molar buffering capacity is

$$\frac{dB}{cdp_H} = 2{\cdot}3\,\frac{k\,.\,[H]}{(k + [H])^2}.$$

So the buffering capacity of the bicarbonate system in final equilibrium at p_H 7 is $2{\cdot}3\,\frac{3{\cdot}10^{-7}\,.\,10^{-7}}{16{\cdot}10^{-14}} = 0{\cdot}43.$

Immediately after addition of an acid, when the system is reacting according to its true constant, we get $2{\cdot}3\,\frac{4{\cdot}10^{-4}\,.\,10^{-7}}{(4{\cdot}10^{-4} + 10^{-7})^2} = 5{\cdot}8 \times 10^{-3}$, a very small buffer value.

Also the second dissociation-constant (6×10^{-11}) gives a very small buffering capacity, viz. $13{\cdot}8 \times 10^{-4}$. It must be concluded, therefore, that immediately after addition of an acid the bicarbonate system cannot exert buffering action at p_H 7.

Preliminary experiments with serum and blood indicate that the presence of free H_2CO_3 after addition of HCl cannot be demonstrated at all. Also there is quite another cause of changes occurring before attainment of equilibrium when HCl is added to ultrafiltrate. These problems will be dealt with in the next paper of this series.

Summary.

1. The change in $[H^+]$ in a bicarbonate solution to which strong acid was added was studied by means of continuous observation of the p_H of the system with an accuracy of 0·005 p_H and a time lag not exceeding $\frac{3}{25}$ sec.

2. The true dissociation-constant of H_2CO_3 and the hydration and dehydration velocities were determined; they appeared to have biologically important values.

3. The velocity of dehydration was shown to be proportional to the $[H^+]$ of the system.

4. At p_H about 7 the bicarbonate system has practically no immediate buffering capacity.

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LXXXII. ON THE DEVELOPMENT OF CHROMOGENIC PROPERTIES IN CHOLESTEROL BY THE ACTION OF HEAT.

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"Oxycholesterol," a brown resinous substance first obtained by Lifschütz [1908] from cholesterol by the action of benzoyl peroxide in acetic acid solution, came into prominence through certain similarities shown by its colour reactions to those associated with vitamin A [Takahashi *et al.*, 1924, 1925]. More recently Rosenheim [1927], working on the arsenious chloride reaction, has also obtained a chromogenic substance from cholesterol by the use of the same reagent, but in chloroform solution, which is found to simulate the "vitamin" colour reactions closely in many respects, though proving unstable in the presence of oils. It is intimated that further experiments are being undertaken using ergosterol, the precursor of vitamin D, in place of cholesterol.

In the meantime certain experiments have come to our notice in which "oxycholesterol" has been formed from cholesterol by other means than the use of benzoyl peroxide or a similar oxidising agent.

(1) It will be recalled that Rosenheim and Webster [1926] suggested that "oxycholesterol" might be formed on the irradiation of cholesterol in air, thus increasing the non-precipitable fraction without raising the antirachitic value. Shear and Kramer [1926] have now confirmed the appearance of this substance under such conditions, having obtained by the prolonged irradiation of cholesterol in air a yellow amorphous product, "U.V. Oil," which gives the typical colour reaction of "oxycholesterol" as described by Lifschütz.

(2) In the course of experiments on the multiplication of infusoria, Robertson [1925], by the aeration of an aqueous colloidal solution of cholesterol at boiling-point in the presence of traces of an acetone extract of brain, has obtained on evaporation a "light brown pasty mass," resembling "oxycholesterol." This material is found to give the typical colour reaction, and is supposed to be produced from the sterol by the action of a catalyst present in the brain extract, in the absence of which no "oxidation" takes place.

In the course of experiments designed to confirm such observations we were led to examine the colour reactions of cholesterol which had been gently heated, both in aqueous colloidal solution and in the solid state. The results stated below would indicate that caution is necessary in accepting the production of "oxycholesterol" as a specific effect of some particular mechanism in cases where heat is also involved.

Experimental.

The experiments were first carried out on a specimen of cholesterol obtained by five recrystallisations from a crude cod-liver oil product supplied by Messrs Joseph Nathan, Ltd. They were repeated on an "almost pure" sample, M.P. 148°, from the same source, obtained by Prof. Heilbron in the course of his recent spectroscopic work, and almost free from ergosterol. Both specimens gave negative results when tested with (1) a 1–5 sulphuric-acetic acid mixture, followed by a drop of 2 % ferric chloride in acetic acid (Lifschütz), or (2) a chloroform solution of antimony trichloride [Carr and Price, 1926], which were the reagents employed to test for "oxycholesterol."

The production of "oxycholesterol" in molten cholesterol.

Samples of cholesterol were heated to temperatures slightly above the melting-point in an open dish on a carefully regulated sand-bath. After some time a slight yellow tint was observed, and on cooling the cholesterol was now found to give positive reactions with the reagents mentioned above, the nature of such reactions depending upon the duration of heating and the temperature attained.

(*a*) If the cholesterol were warmed for a few minutes at a temperature barely above the melting-point a very faint yellow tint was acquired. With the antimony trichloride reagent a blue coloration was given, which faded in a few minutes to a dull brown. With the Lifschütz reagents a preliminary violet was given, which became ultramarine and then greenish blue, but not emerald green, on the addition of ferric chloride. (*b*) If the cholesterol were kept at the same temperature for a longer period (30–120 mins.) the yellow tint developed was more pronounced. A bright ultramarine, permanent for hours, was now given with the antimony trichloride reagent, while in the Lifschütz test a definite green was obtained on the addition of ferric chloride. (*c*) Prolonged heating at more elevated temperatures converted the cholesterol entirely into brown vitreous resins. Some resins gave the complete "oxycholesterol" colour sequence with the Lifschütz reagents, and with antimony trichloride an indigo blue coloration; other resins, in the production of which still higher temperatures were attained, gave no colorations other than brown.

It would seem, therefore, that the production of "oxycholesterol," effected by Shear and Kramer through the agency of ultra-violet radiations, may equally well be effected by thermal means. These workers have also found that irradiated cholesterol gives on boiling with a mixture of 15 parts aniline and 1 part hydrochloric acid a red or reddish brown coloration, which, in spite of strong criticism by Rosenheim and Webster, is still attributed to vitamin D. It may be stated, in support of the view of Rosenheim and Webster, that samples of melted cholesterol were found to react positively to this test, though the original cholesterol gave no coloration. Moreover, samples of a cholesterol,

freed from ergosterol by bromination and reduction, after melting gave positive reactions in this test and in those for "oxycholesterol." The aniline hydrochloride test cannot be considered specific to vitamin D.

The "oxidation" of cholesterol in colloidal solution.

In confirmation of Robertson's work it was found that stable colloidal solutions of cholesterol could be obtained by pouring an alcoholic solution of the latter into hot water, with constant agitation. In our experiments 5 cc. of a 0·5 % solution were poured into 100 cc. of hot distilled water, 105 cc. of solution therefore contained 25 mg. of cholesterol. The solutions so obtained displayed a slight orange opalescence in transmitted light and remained stable for days. As the solutions were not to be used in physiological experiments it was thought unnecessary to follow Robertson's procedure for the removal of alcohol and the solutions were used without further treatment.

After aeration of the colloidal solutions under a reflux condenser at 90–100° for 6 hours with traces of acetone extracts of brain, liver, or kidney, the residues after evaporation gave erratic results when tested with the Lifschütz "oxycholesterol" reagents, the initial colours obtained varying from bright purple to dull brown, which was at first mistaken for a negative reaction. It soon became apparent, however, that the colour produced was entirely dependent on the care taken during evaporation. Cautious evaporation resulted in the production of the normal purple of "oxycholesterol," even in the absence of tissue extracts; overheating in the final stages resulted in a brown colour reaction. Moreover, plain solutions which had not been aerated for 6 hours but merely evaporated carefully to dryness gave positive tests for "oxycholesterol." For a further investigation of the phenomenon it was necessary to evaporate the solutions at a lower temperature. Accordingly they were poured after aeration into large Petri dishes, and left in a hot room at 37° until dry.

Immediate colorations with the Lifschütz and antimony trichloride reagents were given by residues so obtained from solutions heated, without tissue extracts, to 90° for periods down to 1 hour, with or without aeration. No colorations were produced by residues from (*a*) a solution made up and aerated for 48 hours at 37°, (*b*) a solution made up hot and immediately cooled, and (*c*) an alcoholic solution of cholesterol evaporated directly.

A further phenomenon is perhaps worthy of mention. The colloidal cholesterol solutions immediately after preparation were very sensitive to the precipitating action of electrolytes; after heating for some hours they became much less sensitive. Such increased stability would seem to coincide with the formation of the resinous "oxidation" products, but the question has not been investigated in detail.

Discussion.

Cholesterol has long been known to undergo changes on heating. The above experiments show that chromogenic properties appear on such treatment, and consequently in all experiments where the presence or production

of "oxycholesterol" is observed care must be taken to rule out such thermal effects as might arise in the course of the experiment or during the processes of isolation. Thus Lifschütz [1914] has described experiments in which "oxycholesterol" is produced by heating defatted blood with cholesterol in acetic acid solution at 60–65° for several days. While it is possible that the blood acts as a catalyst under these conditions, such evidence does not, in our opinion, justify the assumption of a similar catalytic action in normal physiological processes.

In regard to the experiments of Robertson, as stated above, we have been unable to confirm the reported stability of cholesterol towards "oxidation" when aerated in colloidal solution in the absence of brain extract. Since it is stated that the cholesterol to resist oxidation must be "perfectly pure," we took pains to repeat our experiments on as pure a specimen of cholesterol as was available to us, but still obtained the "oxycholesterol" colour reactions on evaporation. In the absence of a more detailed description of Robertson's experiments we are at a loss to explain this discrepancy.

Cholesterol, of course, may be boiled in most organic solvents without the formation of "oxycholesterol," and the processes of crystallisation and evaporation from such solvents only present danger when excessive temperatures are attained. Assuming that due precautions were taken against this in the experiments of Shear and Kramer all that can be said is that the changes produced in cholesterol by heating and by irradiation in air are superficially very similar.

In conclusion, it should be noted that in our own experiments the cholesterol was usually only in part converted into resinous products, and in some cases only a fraction of such resins must have possessed chromogenic properties. Heat was usually much less efficient in the production of chromogenic substances than the benzoyl peroxide procedure described by Lifschütz.

SUMMARY.

Brown resinous substances are produced in cholesterol on melting or on heating in aqueous colloidal solution. At the same time chromogenic properties similar to those of "oxycholesterol" are acquired.

Our thanks are due to Sir F. G. Hopkins for support and criticism, also to Prof. Heilbron for samples of cholesterol.

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LXXXIII. THE p_H OF THE GASTRO-INTESTINAL TRACT OF CERTAIN RODENTS USED IN FEEDING EXPERIMENTS, AND ITS POSSIBLE SIGNIFICANCE IN RICKETS.

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Although determinations of the p_H of the contents of different parts of the gastro-intestinal tract have been carried out by many previous investigators on such widely different animals as human beings, dogs, cats, pigs, cows and sheep, very little attention appears to have been paid to the rodents so largely used in feeding tests for vitamins. This may be due in part to the greater difficulties involved in handling the minute quantities of material available in such small animals as guinea-pigs, rats and mice. But, in view of the fact that vitamins have already been shown in a number of cases to be affected by the reaction of the medium in which they are present, it seemed to us worth while to investigate the intestinal conditions in these particular rodents.

In the case of the albino rat and vitamin D, interest has been aroused by results recently published by several workers. Zucker and Matzner [1924], working on the faeces only, found that when rats were fed on a rachitogenic diet (Sherman and Pappenheimer's No. 84) the faeces became alkaline (p_H 7·4–8·0), and that administration of vitamin D, either in the form of cod-liver oil, or by direct irradiation of the animals, was followed by a change, within a few days, to p_H 6·0–6·4, although the rachitogenic diet was still continued. After administration of a vitamin D-free cotton-seed oil, this change in faecal reaction from alkalinity to acidity did not take place. These interesting results obtained by Zucker were confirmed by Jephcott and Bacharach [1926], who found that the change followed also after either direct irradiation of the animals or administration of irradiated cholesterol or of the vitamin D fraction of cod-liver oil (ostelin). The faeces, however, represent merely the end of the story. The absorption of calcium and phosphorus, which is closely connected with the rôle of vitamin D, probably takes place mainly in parts of the gastro-intestinal tract where the faecal condition has not yet been reached. It is, therefore, an obvious duty to compare the change in faecal reaction with any

alterations which may simultaneously occur in the p_H of the entire gastro-intestinal tract both on deprivation of vitamin D, and on its readministration to rachitic animals.

An examination of the methods used by other workers indicated some of the obstacles to be expected. It was necessary to collect the material with all precautions possible in order to avoid contamination or undue exposure, and to examine it as soon as possible after the death of the animal. Diet, age, sex, time after last meal, and state of health of the experimental animal were amongst the factors to be considered. Whilst we have attempted to overcome all these difficulties, and numerous others which arose in the course of the work, we realise that the possibility still remains of some unknown factor having been overlooked, and would place this reservation on the results here recorded.

Experimental.

Collection of material.

The animals were killed by chloroform. The abdomen was opened immediately, and the stomach ligatured at both ends. The oesophagus was cut above the ligature, and the large intestine just above the anus. The whole of the gastro-intestinal tract was then removed and measured. Ligatures were applied at each end of the various sections of intestine whose p_H was to be determined. The stomach was rapidly washed with cold water, opened, and the whole of the contents removed and well mixed. A portion was quickly stirred with sufficient distilled water to make a liquid which could be poured into the electrode vessel; a small quantity was set aside for colorimetric estimation, and the remainder triturated with quinhydrone, poured into the electrode vessel, and the p_H determined as described below. The same procedure was adopted with each section of the tract examined, the principle being to avoid contamination by careful washing of the outside of the ligatured section, and to minimise exposure to the air by doing one section at a time, and transferring the mixed material to the closed electrode vessel as quickly as possible. In dealing with the material from the large intestine, when this was of too firm a consistency to mix readily by stirring, it was quickly broken down by rubbing on a glazed porcelain tile with a stiff spatula and just sufficient distilled water to make a thick liquid paste.

Colorimetric estimation.

Colorimetric estimations of the p_H of gastric and duodenal contents have been made by many workers, the methods adopted usually being modifications of Henderson and Palmer's [1912] original technique for estimating the p_H of urine, by comparing the colour given by the material or an aqueous extract on addition of a suitable indicator with the colour given to the same indicator by a standard buffer solution of known p_H. In the case of urine,

which is a fairly stable and well buffered fluid, it is moderately easy to obtain duplicate results agreeing to within 0·1 p_H. When dealing with the contents of the stomach and intestines, however, a number of difficulties arise. In the first place, as has been shown by Kahn and Stokes [1926] in the case of the stomach, and by our own results throughout the gastro-intestinal tract, the material is not usually noticeably buffered until the ileum is reached. Since only minute amounts of material are available from the smaller animals, we were unable to apply Henderson and Palmer's method, which requires the use of a comparator, on account of the risk of dilution. Secondly, as Kahn and Stokes also showed, the most serious difference between colorimetric and electrometric results on gastric contents is due to loss of carbon dioxide, especially in faintly acid or alkaline solutions, and clearing the solution by centrifuging or filtering made very little improvement. Thirdly, there are the well-known protein and salt errors, which may be responsible for differences of 0·2 to 0·5 p_H, but are less serious than the loss of carbon dioxide. On the whole, these sources of error appear to diminish as the material passes down the gastro-intestinal tract, because we usually found widest divergence between colorimetric and electrometric results in the stomach, duodenum and jejunum, less in the ileum, and fairly close agreement in the caecum and large intestine. Our method of colorimetric estimation was as follows. About 0·05 g. of the fresh material was taken up on a standard platinum loop, and mixed on a white glazed porcelain tile with about 0·1 cc. of diluted indicator. If the material was too viscous, it was diluted with two or three times the quantity of distilled water immediately before taking the reading. The colour obtained was compared at once with those given by similar loopfuls of standard buffer solutions of known p_H, mixed each with 1 drop of diluted indicator. The indicators used were thymol blue, bromophenol blue, methyl red, bromocresol purple, bromothymol blue, phenol red and cresol red, as recommended by Clark [1920], but further diluted to about 0·005 %. In every case a change of colour was used, and wherever possible readings were taken on the same sample with two or three different indicators. Duplicate results usually agreed to 0·2 p_H. The method requires the minimum amount of material and of dilution, and involves only a short time of exposure and loss of carbon dioxide. It gave the most satisfactory results in the caecum and large intestine, where the difference between colorimetric results in over a hundred samples was never more than 0·4 p_H, and averaged less than 0·2.

Electrometric estimation.

Electrometric estimations of the p_H in various parts of the small intestine have been carried out by several previous investigators on larger animals. McClendon [1915] and his co-workers, in their numerous experiments on men, cats and dogs, appear to have used generally a special type of hydrogen electrode, and quote results to 0·1 p_H. Kahn and Stokes [1926], using a hydrogen electrode to measure the human gastric p_H, claim to have secured an

accuracy of 0·05 p_H. Long and Fenger [1917], working on material from the stomach and duodenum of men, and the jejunum and ileum of pigs, calves, lambs and a rabbit, and using a hydrogen electrode in a Hasselbalch vessel, quote p_H results to the second decimal place, but do not state what was their experience of the various possible sources of error. We have not been able to discover records of any p_H estimations on the caecum and large intestine. A great deal of work has been done on the faecal p_H in men and different animals. Robinson [1925] compared the results obtained with the hydrogen electrode and the quinhydrone electrode on a series of samples of faeces whose p_H ranged from 5·0 to 7·8, and found the difference to be 0·0 to 0·3, with an average of less than 0·1.

The quinhydrone electrode, since its introduction by Biilmann [1921], has been employed by a number of investigators, and found to give reliable results within the p_H range of 1 to 8. The greatest advantages of the quinhydrone electrode over the hydrogen electrode for this particular work were:

(1) the rapidity and ease with which equilibrium is maintained, as compared with the time required, and difficulty of making readings, when using the hydrogen electrode;

(2) the consequent small loss of carbon dioxide, especially as a current of hydrogen is avoided, and a closed vessel can be used. Three types of electrode vessels were used, one designed by Corran [Corran and Lewis, 1924], and having a gold electrode, and two capillary vessels similar to those recently described by Cullen and Biilmann [1925] in which we used gold-plated platinum electrodes. The formula from which results were calculated was the following:

$$p_H = \frac{0{\cdot}9802 - (0{\cdot}5600 + E)}{0{\cdot}058}$$

E being the observed E.M.F. of the following cell:

$Hg/Hg_2Cl_2/M$ KCl/Saturated KCl/Material saturated with quinhydrone/Au.

After making numerous parallel observations on different samples, we are of the opinion that our average experimental error with the quinhydrone electrode, which was used throughout this work, was less than 0·1 p_H. Although in some of our tables the electrometric results are given to two places of decimals in order to show the variations obtained, we do not consider the second place significant. As will be seen later, a variation from the true p_H of less than 0·1 would not have any important effect on the conclusions to be drawn from our experimental work. When using the capillary electrode vessels, especially the straight tube type requiring only a few drops of liquid, diffusion was minimised by taking the reading as rapidly as possible, as suggested by Biilmann and Lund [1921].

Experiments on guinea-pigs.

Our first experiments were carried out on guinea-pigs, as it was thought advisable to gain experience on moderate-sized animals before turning to the

smaller rats on which the bulk of the work was to be done. The results, however, may be of interest in connection with problems lying outside our own field of work, such as, for instance, the possible relationship between scurvy and acidosis [Lepper and Zilva, 1925].

The guinea-pigs used were normal animals, reared in the Bacteriological Department of the Liverpool City Laboratories. They were fed on a diet of bran, oats, cabbage and water *ad lib.*, and were all in good health. A number of these animals were chloroformed at different times after their daily meal, the gastro-intestinal tract was removed as above described, and the p_H of its contents determined, colorimetrically and electrometrically, at various points throughout its course. It was found that the colorimetric and electrometric results agreed fairly closely (to within 0·2 or 0·3 p_H) when applied to the contents of the ileum, caecum and large intestine, but showed a wide divergence in the duodenum and jejunum, the colorimetric readings always being 0·3 to 1·5 p_H higher than the corresponding electrometric readings. The colorimetric readings on the gastric contents were also higher than the electrometric, but the difference was not so great (0·2–1·0 p_H, average 0·5). This is in agreement with the results obtained by Kahn and Stokes [1926], working on human gastric material.

Table I. *Comparison of results obtained by colorimetric and by electrometric methods for p_H of contents of gastro-intestinal tract of a normal guinea-pig.*

Animal ♂, 6 months old, killed 4 hours after meal.

	p_H	
	Colorimetric	Electrometric
Stomach	4·2–4·4	3·83
Duodenum	6·2–6·5	5·04
Small intestine—		
10–20 cm. below duodenum	6·6–6·8	6·23
30–40 ,, ,,	7·2–7·6	7·70
50–60 ,, ,,	7·3–7·6	7·80
80–90 ,, ,,	7·2–7·4	7·57
Caecum	6·0–6·2	6·37
Large intestine—		
5–10 cm. below caecum	6·2–6·5	6·26
25–30 ,, ,,	6·0–6·2	6·26
35–40 ,, ,,	5·6–5·9	5·74
45–50 ,, ,,	5·6–5·8	5·80
55–60 ,, ,,	5·6–6·0	5·80

In Table I are given details of a typical example, in which estimations were made at as many points as possible. The figures given for the colorimetric results represent the extreme limits reached when each sample was tested with at least two different indicators, and were practically always obtained by independent observers unaware of the electrometric result. The electrometric readings were taken at first in a small electrode vessel whose capacity was about 2 cc. In order to obtain this quantity from the amount of intestinal material available, it was necessary to dilute the latter five to ten times. The

question immediately arose as to what effect this would have on the p_H. An experiment was therefore performed in which longer lengths of the intestine were taken, and sufficient of the contents obtained to make up a series of dilutions at each point. The results are shown in Table II, and from this and a number of similar experiments it would appear that the error due to dilution is greatest in the stomach, duodenum and ileum, and that the gastro-intestinal contents become more effectively buffered as one passes down the tract. On this account we decided to reduce the capacity of our electrode vessel, and after various trials we finally adopted the two types of capillary electrodes previously described, which enabled us to work with 1 in 3 or 1 in 2 dilutions. Judging from a large number of observations on this material in widely different dilutions, including some made with the Kerridge [1925] glass electrode, which permits the use of practically undiluted material, we are of the opinion that our electrometric results obtained with approximately 1 in 3 dilutions do not vary more than 0·1 to 0·2 from the actual p_H of these parts. In the caecum and large intestine the error is probably smaller. (The effect of dilution on the p_H, determined colorimetrically and electrometrically, of faintly alkaline buffered solutions containing varying amounts of sodium chloride has been studied by Lepper and Martin [1926] who found changes of 0·05 to 0·2 p_H when diluting four times.)

We next investigated the effect of chloroforming the animals at different times after the daily meal. Six guinea-pigs were examined, the period varying from $1\frac{1}{2}$ to 17 hours, and the results obtained are summarised in Fig. 1. In plotting these results, the total length of the tract from stomach to anus was taken as a unit, and the various sections of the tract were expressed as fractions of that unit. This enabled an accurate comparison to be made of results obtained on animals of different sizes.

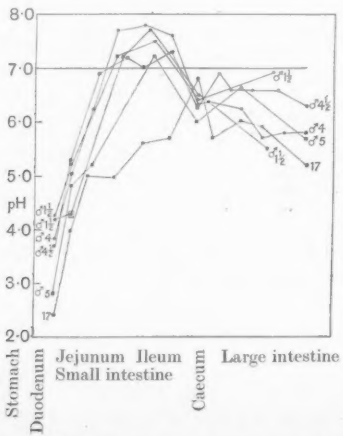

Fig. 1. p_H of gastro-intestinal tract of young normal guinea-pigs.

Table II. *Effect of dilution on the p_H of the contents of the gastro-intestinal tract of a normal guinea-pig.*

Animal ♂, 7 months old, killed 1½ hours after meal.

	p_H			
	Colorimetric	Electrometric		
Dilution	1/3	1/5	1/50	1/250
Stomach	4·8–5·2	4·26	5·04	—
Duodenum	5·8–6·2	5·22	6·62	6·52
Small intestine—				
20–50 cm. below duodenum	6·8–7·2	7·22	6·69	6·61
50–80 „ „	7·4–7·8	7·48	6·93	6·73
Caecum	6·2–6·4	6·41	6·52	6·62
Large intestine—				
30–35 cm. below caecum	6·8–7·0	6·93	6·62	6·52

In general, it was found that the acidity of the contents of the stomach and duodenum tends gradually to increase during the first 4 hours after ingestion of a meal, which is in agreement with the findings of McClendon [1915] on human subjects. In the remainder of the tract, however, no appreciable difference could be observed in the p_H results obtained when the time between feeding and death was anything between 1½ and 4½ hours. Only when food was withheld for as long as 17 hours was there a markedly greater acidity throughout the small intestine. Omitting this abnormal case, the results obtained on the other five animals may be summarised as follows: stomach p_H 2·8–4·3, average 3·8; duodenum 4·8–5·3, average 5·1; jejunum 5·1–7·1; ileum 7·0–7·8, average 7·4; caecum and large intestine 5·2–6·9, average about 6·4. The stomach was always strongly acid, there was a decrease in acidity in the duodenum and ileum, so that the small intestine was mainly alkaline, and the caecum and large intestine, on the other hand, were always acid.

Another point investigated was the effect of entry of bile during dissection. In one animal the common duct was ligatured immediately after opening, and the duodenal contents were found to be more acid (p_H 4·3) than in any of the other four animals. On adding to the duodenal contents 1 cc. of an aqueous extract (about 5 %) of the gall bladder, the p_H was raised to 5·3, the usual value in unligatured animals. These results indicated that the observed p_H of the gastro-intestinal tract might be appreciably affected either by the time of killing, or by the method of dissecting, the experimental animal. The method of killing usually adopted was by chloroforming, but in one or two cases where a blow on the head had been employed no appreciable difference was observed in the p_H results obtained.

Experiments on rats.

Having gained in the experiments on guinea-pigs some knowledge of the special difficulties involved, we next turned our attention to rats, on which the more important work was to be done. Both albinos (inbred from Wistar Institute ancestors) and piebalds were used, and gave similar results. The same technique was employed in preparing the experimental material, and in

taking the colorimetric and electrometric readings. A discrepancy was again observed between the colorimetric and the electrometric readings in the duodenum and ileum. Undue dilution was avoided by the use of the capillary electrodes. The accuracy of these electrodes was tested by taking a number of parallel readings on material from different animals, and it was found that they gave results agreeing to 0·2 p_H or less, the average difference being below 0·1, as shown in Table III.

It was not possible to test the effect of cutting off the supply of bile, because the rat does not possess a gall bladder [Donaldson, 1924], and we were also unable to find the common duct. Whether this hitherto little recognised fact is associated with differences in the digestive processes is a problem for further investigation.

Table III. *Comparison of results obtained with the two types of capillary electrodes, on normal rats.*

Animal	Albino (12 months old)		Piebald (2 months old)	
Electrode	Straight	Side-tube	Straight	Side-tube
		p_H		
Stomach	3·04	3·02	3·97	3·93
Duodenum	6·11	6·11	6·49	6·49
Small intestine—				
Jejunum	6·31	6·50	6·62	6·48
Ileum	6·59	6·79	6·29	6·31
Caecum	6·52	6·36	6·94	7·01
Large intestine	6·50	6·61	6·88	6·99

We then carried out on albinos and piebalds a series of experiments similar to those previously done on guinea-pigs, in which normal animals on different normal diets were chloroformed at different periods after a meal, and the gastro-intestinal p_H determined. The diets fed varied from bread and milk to wheat, maize, oats and kitchen scraps (including greenstuffs), and contained adequate supplies of vitamins. Rats of different ages from 1 to 18 months were used. Typical results are shown in Fig. 2. The curve marked *K* is plotted from results which, by courtesy of Prof. W. C. McC. Lewis, were kindly obtained for us by Dr Millet, using the Kerridge [1925] glass electrode. It will be seen that they agree closely with those obtained by our own method.

In the adult rats little difference was found when feeding these different normal diets. This failure to produce any marked alteration in the intestinal p_H by varying the protein/fat/carbohydrate ratios of normal diets is in agreement with the findings of Abrahamson and Miller [1925], who fed adult albino rats different diets, some consisting largely of carbohydrate (boiled potato), some mainly protein (lean beef), and some mainly fat (beef fat), and obtained on the average only a variation of 0·1–0·2 p_H in the stomach and small intestine. No results were given for the caecum and large intestine, and as their method was entirely colorimetric, the possibility of significant errors cannot be ignored. Their colorimetric results on a series of 36 animals were: stomach 2·6–4·1, average 3·9; duodenum and jejunum 5·2–6·4, average 5·8;

ileum 5·2–6·0, average 5·8. Our electrometric results on a series of 9 adult animals were: stomach 3·0–5·4, average 4·2; duodenum 4·0–5·7, average 5·2; jejunum 5·0–6·3, average 5·7; ileum 5·7–6·6, average 6·4; caecum 6·1–6·9, average 6·4; large intestine 6·9–6·9, average 6·4. As in the guinea-pig, the effect of varying the time between last meal and death was most marked in the stomach, and appeared to produce little difference elsewhere.

Half a dozen young normal rats (4–12 weeks old) on different normal diets were also examined. Typical results are indicated in Fig. 2 by broken lines, the age of each animal also being given. (In order to avoid complicating the figures, only a certain number of typical animals are included.) It will be seen that in young rats the small intestine apparently tends to be more alkaline than in adults (jejunum 6·5–7·2, average 6·8; ileum 6·3–7·6, average 7·0), but that the caecum and large intestine were always acid (6·5–6·9, average 6·7). From the recent work of Bergeim [1926] it appears that these last two sections of the gastro-intestinal tract are probably the most important in regard to calcium metabolism and the action of vitamin D.

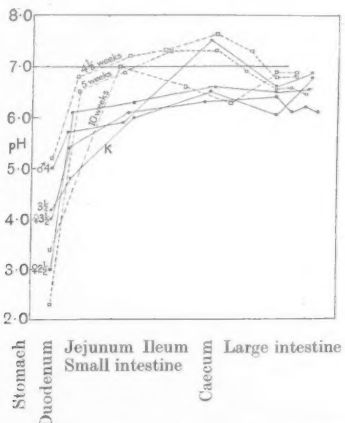

Fig. 2. p_H of gastro-intestinal tract of normal rats of different ages.
Young rats: – – – ▫ – – –. Adult rats: ——— ▪ ———.
The figure against each curve represents the number of hours between last meal and death.

Another question which arises is whether the p_H of the gastro-intestinal tract is in any way influenced by the nature of the intestinal bacterial flora. This problem has been carefully studied by Rettger and Cheplin [1921], working on both albino rats and human beings, and in both cases the conclusion was reached that the diet is the controlling factor. In regard to rickets, moreover, data obtained in experiments on dogs with various organisms isolated from the intestines of rachitic subjects would seem to render it unlikely that a bacteriological factor is predominant [Findlay, 1926]. It seemed to us that the fundamental fact to be investigated was the change in

faecal p_H which Zucker has found to be produced by the action of vitamin D, and, having found that in normal rats the p_H in the caecum and large intestine was always below 7·0, we decided next to examine the effect of withholding this vitamin.

An examination of the diets used by various workers to produce experimental rickets in rats showed that these can be classified in two main groups, according to their content of carbohydrate and fat. One, which has been employed in America by Sherman and Pappenheimer, Zucker and others is noticeable for its high carbohydrate content (nearly 70 %), and especially for its very low proportion of fat (less than 3 %) (see Table IV). The other, which has been principally used by British workers, contains rather less carbohydrate (50–60 %), and much more fat (15–17 %), and resembles much more closely in general composition the natural diet of infants. Since all the experiments so far recorded on the reaction of the faeces in rickets have been done on rats receiving the first "low fat" type of diet, we thought it would be of interest to ascertain if the change of faecal reaction to alkaline would also be obtained on withholding vitamin D from rats on a "high fat" diet.

Table IV. *Composition of diets used to test for vitamin D.*

	"Low fat" type	"High fat" type
Carbohydrate—		
Wheat starch (in flour)	69	—
Potato starch	—	56
Protein—		
Egg albumin and wheat protein	21·6	—
Caseinogen	—	23
Fat—		
Natural fat in wheat flour	1·7	—
Inactivated palm kernel oil	—	17
Salts—		
Calcium lactate	2·8	2·7
Ferric citrate	2·0	0·2
Sodium chloride	2·0	0·2
Other minerals	see note below	1·9
	99·1	99·0

Some workers use wheat starch in their "high fat" diets.

In certain "low fat" diets the protein is partly supplied as gelatin and maize, and the fat content may be a little higher (between 2 and 3 %).

Palm kernel oil is replaced by inactivated cotton-seed oil in certain "high fat" diets.

Variable amounts of phosphates of calcium, magnesium and potassium, and traces of silica and other minerals are present in wheat flour. The "low fat" diets are usually noticeable for containing a high percentage of sodium chloride (1 to 2 %).

The above figures are calculated on dry materials.

Vitamin A is usually supplied as spinach.

Vitamin B in the "low fat" diets is present in the cereal.

Vitamin C in the "high fat" diets is supplied as lemon juice.

In most of the previous studies on experimental rickets no account has been taken of the differentiation of the fat-soluble vitamins, and the animals have been deprived of vitamin A as well as of vitamin D. Chick and Roscoe [1926] appear to have been the first British workers to investigate the effect of depriving albino rats of vitamin D only, vitamin A being supplied daily in the form of fresh spring or early summer spinach leaves. The two diets

used were of the "high fat" type (15 and 17 %), and had calcium/phosphorus ratios of 0·4 : 1 and 0·7 : 1 respectively. No record was made of the intestinal reaction or the faecal p_H. Slight rickets was produced, the degree of calcification being nearer to normal than in animals also deprived of vitamin A. In these experiments, however, Chick and Roscoe were not always successful in avoiding xerophthalmia by the use of fresh spinach leaves, although the daily dose in some cases reached nearly 3 g. In view of these results, we decided to make certain modifications in Chick and Roscoe's technique when planning a similar investigation, in which the intestinal condition was also to be observed, and the relationship studied between faecal reaction and calcium and phosphorus retention. To ensure a satisfactory supply of vitamin A, whilst avoiding unduly bulky doses of spinach leaves, we prepared an acetone-ether extract of freshly dried early summer spinach, and have up to the present been able to secure freedom from xerophthalmia in all our experimental animals by supplying this extract in 25 mg. daily doses as the sole source of vitamin A. Further tests which are being carried out appear to indicate that 50 or 100 mg. doses of the extract do not contain significant amounts of vitamin D. In addition to using this spinach extract in place of the fresh leaves, we have employed a "high fat" type of diet whose calcium/phosphorus ratio more closely approaches the value 1·6 : 1 which is considered by McCollum [1923] to be approximately optimal for calcium absorption [cf. Willimott and Wokes, 1926, 1]. We hope to be able to publish later details of these studies.

The point of interest now is that when we had removed as far as possible all abnormal factors by supplying adequate amounts of vitamin A, and by using a diet with a normal fat content and a practically normal calcium/phosphorus ratio, we have still found that deprivation of vitamin D produces an alkaline faecal reaction, which changes back to acid on restoration of the vitamin. Daily tests over a number of weeks on the vitamin D-free diet have given an average faecal p_H of 7·3, with fluctuations from 6·5 to 7·8.

In order to satisfy ourselves that the basal diet itself is not responsible for producing the alkaline condition, we have fed it to normal rats receiving adequate supplies of vitamins A and D, vitamin B being supplied in the form of a yeast extract, and have examined the intestinal reaction after different periods. The results, which are shown in Fig. 3, would seem to prove that the diet tends to produce in the gastro-intestinal tract a condition which is more acid than normal, probably on account of the type of salt mixture employed.

In regard to the faecal p_H in normal rats, it is of interest to note that Rettger and Cheplin [1921] when examining 146 samples from albinos on different diets, found them always acid.

We have also paid attention to the "low fat" type of diet. Thirteen different young albinos and piebalds which were fed this diet (see Table IV) developed a definitely alkaline faecal reaction (p_H 7·3–8·0) in 10 to 14 days. Three of these animals, when their faeces had remained at about p_H 8·0 for at least a week, were chloroformed and examined. The results, which are

shown as continuous lines in Fig. 4, indicate a definite tendency towards greater alkalinity throughout the tract. Even the stomach, after digestive activity had subsided, was only faintly acid (p_H 6·1–6·5). In one animal the most alkaline point reached was only 7·2, but in the other two a reading of 7·8 was recorded. At the lower end of the large intestine these two gave slightly higher readings (7·3 and 7·4) than the less alkaline animal (7·2), so that, judging by these three cases, the p_H of the faeces in rats on Zucker's diet would appear to give a fairly reliable indication of the conditions higher up the tract, in those parts where calcium absorption is probably most important. Abrahamson and Miller [1925], using colorimetric methods, also found that deprivation of vitamin D raised the p_H from 6·0 to 7·0 in the jejunum, and from 6·0 to 7·1 in the ileum. Unfortunately the colorimetric results were not checked by electrometric methods, and no readings were taken in the caecum or large intestine. On administration of cod-liver oil, however, the original acid condition was restored.

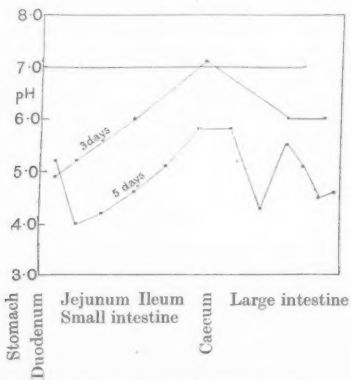

Fig. 3. Effect of "high fat" type of rachitogenic diet on p_H of gastro-intestinal tract of normal rats receiving vitamin D.

Autopsies on our three rats did not reveal any marked signs of rickets, which was perhaps not surprising in view of the short period they had been on the rachitogenic diet. Microscopic and chemical examinations of bones from animals which have been kept on the diet for a longer period are now being made, and would appear to indicate the incipient stages of the disease. The condition of the animals is, in general, very similar to that which McCollum obtains in the preparatory period for his "line" test [McCollum, Simmonds, Shipley and Park, 1922].

On administering to the animals whose faecal p_H had remained at about 8·0 for 14 days vitamin D as cod-liver oil or as irradiated cholesterol, we found that the faecal p_H dropped gradually to about 6·0. The rate at which it drops seems to be influenced by the amount of vitamin D supplied, but if this is too large the faeces become very loose. In the case of four animals which had

been on exactly the same treatment, for the same period, administration of the same amount (about 0·15 g.) of a Norwegian oil brought the p_H down at approximately the same rate, the greatest variation in p_H of faeces (which were collected separately from each animal) on any day being 0·5. Taking the faeces of any one animal on any given day, the greatest variation we have found in a number of samples is 0·2, and often it is much less. For this reason we suggest that an accuracy of 0·1 in the p_H determination is sufficient for the Zucker method.

We have also tested the effect of adding to the Zucker diet a well buffered natural food substance. For this purpose we used the freshly expressed juice of ripe Californian navel oranges (Sunkist brand). This had an average p_H of 4·2, and was so well buffered that 12 to 15 cc. of N NaOH were required to bring 100 cc. to p_H 7. Four albino rats were put on a Zucker diet, and vitamin A was supplied as a daily ration of 2–3 g. fresh spinach leaves. When the faeces had been alkaline for over a week, each animal was then given a daily dose of 5 cc. of fresh orange juice. Since one of us had found this amount of juice to contain adequate amounts of vitamin A (unpublished data) the spinach was then discontinued.

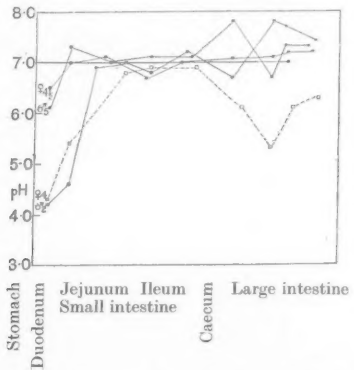

Fig. 4. Effect of vitamin D on p_H of gastro-intestinal tract of young albino rats on Zucker's "low fat" diet.

Rats on vitamin D-free diet: ——— ▪ ———. Rats receiving D: – – – ▫ – – –.
The figure against each curve represents number of hours between last meal and death.

Daily readings taken for over a fortnight showed that the faecal p_H still remained alkaline (7·5–8·0). Vitamin D was then administered in two different ways, as irradiated cholesterol and as cod-liver oil, and in each case the faeces gradually became acid. One of the animals, when its faeces had been acid for 5 days, was then chloroformed and examined. The results, which are shown as broken lines in Fig. 4, indicate that administration of vitamin D had restored the acid condition throughout the gastro-intestinal tract.

Experiments on other rodents.

Mice are not frequently used in feeding experiments on vitamins, although they were employed in the pioneer investigations of Stepp [1909]. Some white mice kindly supplied by Sir F. G. Hopkins were examined. The results would seem to show that the reaction in the gastro-intestinal tract of the white mouse on a bread and milk diet is similar to that of the rat, but somewhat more acid, the highest p_H recorded being under 6·0. The amounts of material available, however, were so small that the results are not considered sufficiently accurate for publication of details.

Rabbits have recently been used by Mellanby and Killick [1926] as experimental animals for the production of rickets, being fed on a basal diet of bran and oats, with turnip to supply vitamin C. We carried out an experiment on a young rabbit which had been fed on a diet of bran and oats, with cabbage to supply vitamins A and C. The results, which are summarised in Fig. 5, resemble those previously obtained on the guinea-pig, another herbivorous animal.

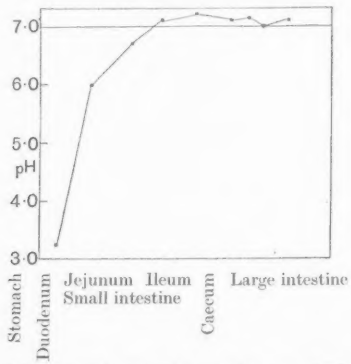

Fig. 5. p_H of the gastro-intestinal tract of a normal adult rabbit.

Discussion.

Although this investigation is in various respects incomplete, we think it desirable to mention certain points which may help to elucidate our experimental results, and perhaps indicate new lines of attack. Dealing first with the rat, on which the bulk of our work has been done, we have shown that deprivation of vitamin D produces in the gastro-intestinal tract a greater tendency towards alkalinity, and restoration of the vitamin brings the reaction back to acid. In the caecum and large intestine the difference between rats receiving vitamin D, and those deprived of it, is even more marked, a change in p_H of such a small range as 6·6 to 7·4 appearing to be significant. Assuming for the moment that the incidence of rickets is influenced by the degree of calcium absorption in the intestines, it is obvious that this in turn will be subject to various factors which affect the solubility of calcium. Under normal

conditions, the gastric hydrochloric acid probably is sufficient to render into soluble form practically the whole of the calcium in the food, as has been shown by Telfer [1924] in experiments both *in vitro* and *in vivo*, using dogs as subjects. As the food passes down the intestine, the gastric acidity tends to be more or less neutralised by the bile and other alkaline secretions, and calcium may be precipitated as carbonate, as soaps or as phosphates [cf. Willimott and Wokes, 1926, 2]. Blauberg [1900] has published analyses of the faeces of rachitic children, indicating that in rickets an abnormal amount of calcium is excreted in combination with phosphorus. Robison [1923] has demonstrated the presence in the wall of the small intestine in rats, guinea-pigs and rabbits of the phosphoric esterase which he suggests performs an essential part in calcification in the bones by liberating phosphate ions, and thus precipitating calcium phosphate. Robison and Soames [1924], using a preparation of this enzyme to hydrolyse different phosphoric esters, have shown that it has an optimum p_H range of 8·4–9·4, and rapidly loses its activity as the p_H falls from 7·5 to 6·8. Provided that this enzyme is present in the intestine, the suggestion arises that the change in faecal and intestinal reaction from alkaline (p_H 7·2–7·8) to acid (p_H 6·5–6·8) which we have shown to occur in rachitic rats after administration of an antirachitic substance, may, by retarding the action of this phosphoric esterase, diminish the precipitation of calcium phosphate and thus facilitate calcium absorption from the intestines. If such is the case, then a metabolism experiment should show that the calcium retention is greatest when the faeces are acid, and least when they are alkaline, provided that the reaction of the faeces is a reliable indication of the p_H of the parts of the intestines most particularly concerned with calcium absorption and retention. In regard to the latter point, the recent results of Bergeim [1926] are significant. By means of experiments on rats, he found that on a rachitogenic diet the absorption of calcium is most seriously affected in the caecum and large intestine, where it might even become negative.

When vitamin D was administered in the form of cod-liver oil, at the level of 10 drops a day, the following increases were observed in the average percentage absorption of calcium: in the jejunum, an increase from 63 to 73, in the ileum 35 to 57, in the caecum nil to 50, in the large intestine minus 5 to 80. It is interesting to compare these results, which were obtained on a "low fat" type of diet, with the intestinal p_H we found in the three rats on the Zucker "low fat" rachitogenic diet. In all three cases the most alkaline point reached was not until the lower end of the small intestine, and in two cases there was only significant alkalinity (p_H 7·2–7·8) in the caecum and large intestine.

These results having led us to suppose that the faecal p_H can be considered a reliable indication of the intestinal reaction, we have set in train metabolism experiments in which the calcium and phosphorus retentions are being measured in rats fed different diets deficient in vitamin D, and compared with daily variations in the faecal p_H. We hope to be able to publish later details of our findings.

In regard to guinea-pigs, the fact that we have shown that the greater portion of the small intestine is normally definitely alkaline (p_H 7·2–7·8), while the caecum and large intestine are acid (p_H 6·8–5·2), would seem to suggest that in this animal also a significant amount of calcium absorption takes place in the caecum and large intestine.

In regard to human beings, Flamini [1926] has recently reported results of experiments in which he found that the administration of vitamin D concentrate (ostelin) to rachitic babies was followed by a change in the reaction of the faeces from alkaline to acid, the "titratable acidity" for 100 cc. of faeces altering from 100–150 cc. $N/10$ H_2SO_4 to 100–300 cc. $N/10$ NaOH. Observations are now being made with the object of ascertaining if the reaction of the faeces in children can be correlated with the rachitic condition as it apparently can be in rats.

Summary.

Colorimetric and electrometric methods for determining the p_H of the gastro-intestinal tract were examined, and an estimate made of the errors due to protein and salts, dilution and loss of CO_2. A satisfactory technique was evolved using a capillary electrode vessel.

In guinea-pigs the following average p_H results were obtained: stomach 2·8–4·3, average 3·8; duodenum 4·8–5·3, average 5·1; jejunum 5·1–7·1; ileum 7·0–7·9, average 7·4; caecum and large intestine 5·2–6·9, average about 6·4.

In normal adult rats (albinos and piebalds) the average figures were: stomach 4·0, duodenum 5·5, jejunum 6·0, ileum 6·8, caecum and large intestine 6·5.

In rats 1–3 months old the average figures were: jejunum 6·8, ileum 7·0, caecum and large intestine 6·7.

A "high fat" type of rachitogenic diet when fed to rats gave greater acidity to the faeces and throughout the intestinal tract if vitamin D was supplied, but in the absence of vitamin D the faecal reaction became alkaline.

Rats fed on Zucker's "low fat" rachitogenic diet developed alkalinity throughout the intestinal tract, but especially in the caecum and large intestine (average p_H 7·3), which from Bergeim's work would appear to be the most significant areas in calcium absorption and retention. Administration of vitamin D as cod-liver oil or as irradiated cholesterol restored the acidity.

On account of increased buffering in the caecum and large intestine, the p_H of the faeces, which has been suggested as a criterion for the estimation of vitamin D, would appear to be a reliable indication of the reaction in these sections of the gastro-intestinal tract, at least in the case of the rat.

Zucker's method for testing for vitamin D has been applied to a buffered natural food substance (orange juice).

The mouse and rabbit were also examined.

A possible relationship is discussed between the intestinal p_H and the occurrence of a phosphoric esterase in the aetiology of rickets.

We are indebted to Sir F. G. Hopkins for his advice and interest in the work, to Prof. J. M. Beattie for laboratory facilities, and also for kindly diagnosing the condition of the animals, and to the scientific staff of Messrs Joseph Nathan and Co. for affording us free access to their experimental results.

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LXXXIV. STUDIES IN THE SULPHUR METABOLISM OF THE DOG.

V. TOXIC ACTION OF MERCAPTURIC ACIDS.

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From the Biochemical Laboratory, Cambridge.

(*Received May 2nd, 1927.*)

So far as has been ascertained there are only two records in the literature of the administration of mercapturic acids to dogs. Baumann and Preusse [1881] administered by mouth 8 g. of the ammonium salt of *p*-bromophenylmercapturic acid. No ill effects were observed; no glycuronic acid was excreted and only traces of mercapturic acid. No sulphur analyses apparently were done. Marriott and Wolf [1907] gave 1 g. of the bromo-acid by mouth. No ill effects were recorded but in the record the sulphur analyses of the urine do not show clearly whether any mercapturic acid was excreted. Possibly absorption was incomplete in both cases.

In the year 1922 during the course of experiments recorded in Part III of this series [Callow and Hele, 1926] it was found quite unexpectedly that an oral dose of 1 g. of *p*-bromophenylmercapturic acid produced a transient haemoglobinuria in the dog "Vixen." This result has been confirmed in several other dogs.

Experimental.

The methods employed in this research were identical with those already described [Callow and Hele, 1926]. Five dogs were used. "Vixen" and "Patricia" received the diets mentioned previously. "Diana" was not placed on a standard diet. "Phoena" and "Pansy" were given the following diets:

"Phoena" (6·97 kg.):

		T.N. g.	T.S. g.	Calories K
Lean meat	80 g.	2·73	0·181	106
Margarin	40 g.	—	—	312
Arrowroot biscuit	20 g.	0·22	0·025	121
Milk	160 cc.	0·83	0·074	115
	Total	3·78	0·280	654

"Pansy" (8·62 kg.):

		T.N. g.	T.S. g.	Calories K
Lean meat	80 g.	2·73	0·181	106
Margarin	20 g.	—	—	156
Sucrose	50 g.	—	—	205
Milk	160 cc.	0·83	0·074	115
	Total	3·56	0·255	582

Exp. 1. "Vixen." At 11 a.m. 1 g. *p*-bromophenylmercapturic acid was administered by mouth. At 5 p.m. on the same day the urine voided was almost black in colour and contained much haemoglobin. The microscope showed the sediment to consist of red-cell debris. There was evidence neither of haematuria nor of a primary nephritis. At 5 p.m. on the following day the urine was free from haemoglobin. Traces of albumin however persisted for another two days.

Exp. 2. "Patricia." 0·5 g. *p*-bromophenylmercapturic acid was administered by mouth. No ill effects were noted. The dose was too small to make any appreciable alteration in the output of neutral sulphur.

Exp. 3. "Phoena." At 11 a.m. 2·5 g. *p*-chlorophenylmercapturic acid were administered by mouth. At 9 a.m. on the following day 15 cc. of deep red urine were removed from the bladder, the previous urine being clear. The red urine contained much free haemoglobin, much cell debris and a few blood cells. At 12.45 p.m. there was in the urine only a trace of haemoglobin with a few blood cells. Until 9 p.m. there persisted a trace of albumin but no further trace of haemoglobin. The dog vomited the evening meal on the first day and afterwards refused all food except the meat and milk. In consequence the outputs of nitrogen and of neutral sulphur, which rose considerably, had no experimental value. There was however no increase in the outputs of ethereal sulphate, free or conjugated phenols, indicating that there had been no conversion of mercapturic acid to ethereal sulphate. The "organic" chlorine excreted in the first 24 hours after the dose corresponded to only 6 % of the dose, but on the following day during the recovery period the organic chlorine indicated an excretion of 30 % of the dose. Although chlorides were freely excreted during the haemoglobin period yet there was a marked retention of nitrogen. Afterwards the chloride excretion fell and the nitrogen rose. The excretion of sulphate was similar to that of the nitrogen. Fig. 1 shows graphically the excretives in the urine during the experimental period. Fig. 2 shows the concentration of chloride (expressed as chlorine), total nitrogen and sulphate in the urine and also the variations in its volume, associated with variations in the water intake.

Exp. 4. "Diana." 0·5 g. *p*-chlorophenylmercapturic acid was given subcutaneously as the ammonium salt. No ill effects were noted.

A week later 1 g. *p*-bromophenylmercapturic acid was given subcutaneously as the ammonium salt at 10 a.m. At 10 p.m. there was a trace of haemoglobin in the urine. The urine voided during the night was very dark coloured and the haemoglobin persisted until 2.30 p.m. on the following day. After 4.30 p.m. the urine contained no albumin. During the haemoglobin period the sediment contained much cell debris with a very occasional blood cell or epithelial cell. There were no casts until the recovery period and then only a few epithelial and blood casts.

Exp. 5. "Pansy." This experiment is shown in Fig. 3. At 10 a.m. 1·23 g. *p*-bromophenylmercapturic acid was administered by the mouth. The urine

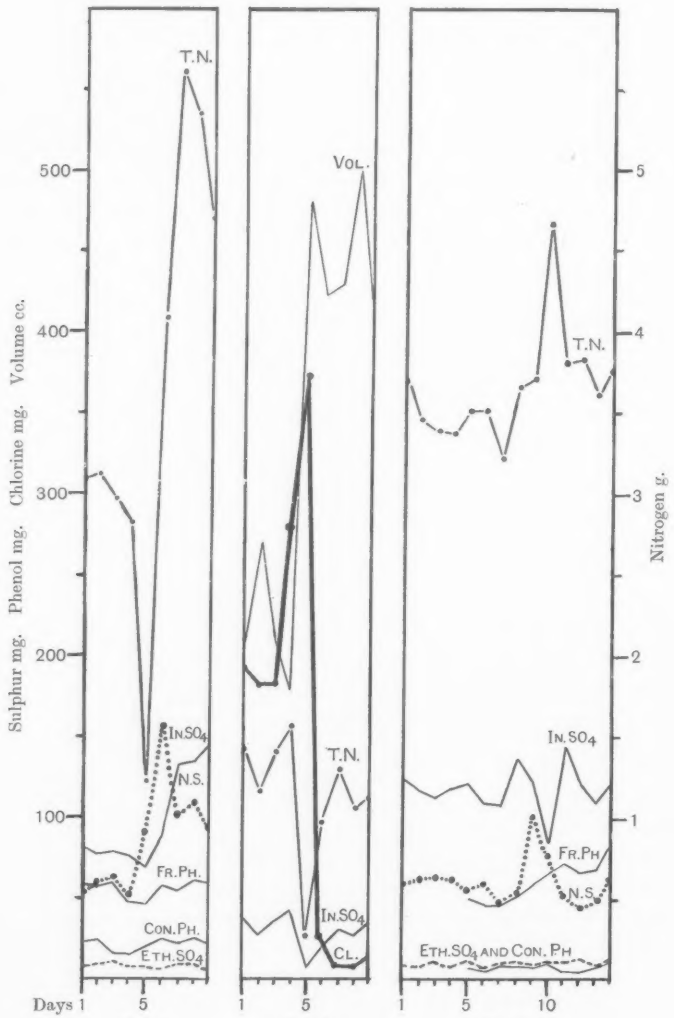

Fig. 1. *Exp.* 3.

Fig. 2. *Exp.* 3.

Fig. 3. *Exp.* 5.

Figs. 1 and 2. "Phoena." Day No. 5. 2·5 g. *p*-chlorophenylmercapturic acid.
Fig. 3. "Pansy." „ No. 9. 1·23 g. *p*-bromophenylmercapturic acid.

remained clear until 5 p.m., when the dog was placed in the metabolism cage. The urine passed during the night was mostly clear, though there was a little blood at the bottom of the collecting vessel. At 9 a.m. on the following day 17 cc. of bloody urine were removed from the bladder. This urine contained much cell debris, only a few red cells and no casts. For the quantitative work this urine was discarded. For the next 24 hours the urine was cloudy, containing traces of blood and some albumin. On the third day the urine was clear and free from albumin. The diet was maintained throughout, so that variations in the quantities of the urinary products could not be ascribed to errors of diet. During the "albumin" period estimations in duplicate were carried out on the untreated urine, on the urine rendered protein-free by trichloroacetic acid and on the urine rendered protein-free by "dialysed iron." The agreement between these was very close, showing that the quantity of albumin present was too small to affect the neutral sulphur figure appreciably. The rise in neutral sulphur corresponded to an excretion of 32 % of the dose in the first 24 hours and 16 % in the second. There was no retention of nitrogen as in Exp. 3, showing that the permeability of the kidney was hardly affected. There was no alteration in the outputs of ethereal sulphate or conjugated phenols, indicating that there had been no conversion of mercapturic acid to ethereal sulphate. No explanation is offered for the rise in the output of nitrogen on the tenth day and of inorganic sulphate on the eleventh day. The rise in free phenols was slow and continuous and bore no relation to the dose-day.

Discussion.

From the above experiments it is clear that the administration of *p*-bromo- or chloro-phenylmercapturic acid may produce a transient haemoglobinuria in the dog. There is a minimum dose necessary to bring about this effect. If the haemoglobinuria is considerable and the kidney tubules are largely blocked with cell debris, there is a retention of nitrogen and the excretion of the mercapturic acid is delayed. These experiments would suggest that this effect of mercapturic acid might be used as a method of producing experimentally a variable degree of "blocked kidney," of the kind observed in blackwater fever. The cause of the haemolysis which precedes the excretion of the haemoglobin is not clear. The effect is observed, whether the mercapturic acid is administered orally or hypodermically. Preliminary experiments on the direct action of mercapturic acids on the blood cells of the dog *in vitro* suggest that mercapturic acids have no direct haemolysing action. The action of mercapturic acids is remarkable, when the comparatively non-toxic character of bromo- and chloro-benzene is considered. These dogs are capable of dealing with 5 g. of bromobenzene in a single dose without haemolysis being produced, that is ten times the dose of bromobenzene equivalent to the toxic dose of the corresponding mercapturic acid. Of course the concentration of mercapturic acid in the effective organ may never be so high

at any given moment after bromobenzene as after the administration of the mercapturic acid. Until, however, something is known about the mechanism of the production of the haemolysis, it would be wrong to regard the formation of mercapturic acid in the dog as a protective synthesis.

It must be noted that in these experiments there is no indication of any conversion of mercapturic acid to ethereal sulphate. Any such conversion would be seen in the outputs of ethereal sulphate and of conjugated phenols.

SUMMARY.

1. *p*-Bromo- and *p*-chloro-phenylmercapturic acids, whether given by the mouth or subcutaneously, cause a transient haemoglobinuria in the dog. There is a minimum dose required to produce this effect.

2. There is no evidence of any conversion of mercapturic acids to ethereal sulphates in the dog.

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LXXXV. STUDIES IN THE SULPHUR METABOLISM OF THE DOG.

VI. THE METABOLISM OF THE PIG AND DOG COMPARED[1].

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(*Received May 2nd, 1927.*)

In a former communication one of us [Hele, 1924, 1] was able to show that ethereal sulphates are formed by direct union of phenolic compounds with preformed sulphate. This work is directly opposed to the views held by Shiple, Muldoon and Sherwin [1924], who have published an account of work, which they interpret as evidence of the formation of ethereal sulphates from some product of endogenous sulphur metabolism, something of the type of a mercapturic acid being the intermediary. It seemed to us that perhaps this difference of opinion was caused by a difference in the species of animal employed, because, whereas dogs were used in this laboratory, a pig was used in the experiments of Shiple, Muldoon and Sherwin. We therefore decided to study the metabolism of the pig and to repeat as far as possible the experiments, which have been described in the former papers of this series and in which the dog was used as the experimental animal.

Early in our work it became obvious that there are great differences in the metabolism of the pig and of the dog. We found that the pig, unlike the dog, does not readily detoxicate phenolic substances by combination with compounds containing sulphur. The increase in the reducing power of the urine would suggest that the detoxicating agent is glycuronic acid. This then we believe to be the essential difference. Whereas in the dog ethereal sulphates and mercapturic acids are formed readily and glycuronic acids less readily, in the pig glycuronic acids are formed readily, ethereal sulphates less readily and mercapturic acids probably not at all. When our work was extended to the metabolism of the mercapturic acids, we also found a difference between the two species. No evidence could be obtained of a conversion of mercapturic acids to ethereal sulphates. In this respect the pig resembles the dog. On the other hand, with the doses used, mercapturic acids produced no sign of the haemoglobinuria which is so remarkable in the dog.

[1] The previous papers of this series are numbered 1—5 in the list of references.

Experimental.

Two experiments were carried out on different animals, pure bred female pigs, recently weaned, of the "Large White" breed. Young pigs had to be used, as there were no facilities in the laboratory for using adult pigs. An attempt was made to catheterise the pigs daily, a procedure so valuable in metabolism experiments on the dog. In the young pig this operation is difficult and was soon abandoned in favour of changing the pig from cage to cage every few hours, as each cage became fouled. The 24-hours' urine was made up each morning at 9 a.m. The volume was always large (1500 to 2000 cc. as compared with the 200 to 300 cc. of the dog). We found the pig's habits to be very regular and as any error in collection would be of urine passed many hours after food and therefore of low sulphur content, we thought failure to catheterise was not a serious matter. The analytical figures obtained justified this practice.

The diets used were as follows:

Exp. 1 (Fig. 1):

"Phyllis," born May 8th. Experiment July 16th to August 23rd, 1926. Weight at commencement of experiment, 14·5 kg.; at end, 18·5 kg.

9 a.m.	Sharps 175 g.	Water 1000 cc.
12 noon	Grey peas 200 g.	Fed dry
5 p.m.	Sharps 175 g.	Water 1000 cc.

The nitrogen content of the diet was 15·4 g.

Exp. 2 (Fig. 2):

"Rosamund," born July 23. Experiment October 29th to November 20th, 1926. Weight at commencement of experiment, 17·7 kg.; at end, 18·6 kg.

9 a.m.	Sharps 250 g.	Water 1500 cc., cabbage 100 g.
12 noon	Grey peas 200 g.	Fed dry
5 p.m.	Sharps 250 g.	Water 1500 cc., cabbage 100 g.

The nitrogen content of the diet was 19·6 g.

The "Sharps" were soaked for 24 hours in half the ration of water and the remainder of the water was added at the time of feeding. The sodium sulphate and guaiacol carbonate used in Exp. 1 were mixed with the morning and evening food. The remaining substances were administered in gelatin capsules. At first we had considerable difficulty in getting the pig to swallow the capsules but later, using the following technique, we were able to give the various doses easily and with certainty.

The pig is held with the fore feet off the ground between the knees of an assistant, who is in a standing position. A piece of thin rope is tied round the upper jaw behind the snout and the snout is drawn upwards and backwards. Under these circumstances the pig keeps the mouth open. The operator then draws the tongue forward preferably with forceps and throws or blows through a tube the capsule to the back of the tongue. The tension on the rope is immediately released and the pig swallows the capsule. The assistant should place a stick between the teeth on one side of the pig's mouth to prevent it accidentally biting the operator. If available a second assistant can be employed in holding the pig's back legs off the ground.

All the chemicals that were administered were of the highest degree of purity. The guaiacol carbonate, phenol, bromobenzene and iodobenzene were obtained from Kahlbaum, the *p*-iodophenol was made from *p*-aminophenol by the method of Brazier and McCombie [1912] and the mercapturic acids were prepared from the urine of dogs by the usual method.

The analytical methods have all been previously described [Hele, 1924, 2] except those for the determination of iodine. For this the method described by Kendall [1920] proved very satisfactory. We preferred however to incinerate with sodium peroxide instead of with sodium hydroxide.

The formation of ethereal sulphate.

We tried to repeat the experiments described in the first paper of this series [Hele, 1924, 1], using the pig in place of the dog. Larger doses were used of sodium sulphate (4 g. daily) and of guaiacol carbonate (10 g. on each of two days), as the normal output of inorganic sulphate was larger in the pig (Exp. 1, Fig. 1, days 5 to 13). About 80 % of the sulphate was absorbed and excreted. The guaiacol carbonate, however, gave rise to only a minute amount of ethereal sulphate, the excess over the normal daily output of ethereal sulphate-sulphur on the two days being respectively 14 and 27 mg. S and on the day following 34 mg. S. Estimation of the phenolic substances in the urine by the colorimetric method [Folin and Denis, 1915, 1916] suggested that there were being excreted each day about 2 g. of the guaiacol, equivalent to about 500 mg. sulphur. As guaiacol is rather insoluble, we tried phenol (3 g., Fig. 1, day 11). The excess ethereal sulphate-sulphur on the phenol day was only 74 mg. In the dog this dose of phenol would have yielded an excess of at least 400 mg. The colorimetric method indicated an excretion of about 2 g. of the phenol in the urine, equivalent to 680 mg. S.

It therefore became obvious that the experiment, which proved so successful on the dog, could not be repeated on the pig, because so small an amount of ethereal sulphate was formed in the latter case. To prove the point at issue it is not sufficient merely to produce a rise in ethereal sulphate larger than the rise following the administration of the phenol alone, but it is necessary to produce such a rise that it is immediately clear that some of the administered sulphate has been utilised. It would have required at least 18 g. phenol, too large a dose for safety, to convert all the inorganic sulphate of food and tissue origin and some of the administered sulphate to ethereal sulphate. Quite apart from any direct action on sulphur metabolism such a dose, even if given successfully, would probably have caused an increase in tissue breakdown. The rise in the output of sulphate from this source would then have obscured the issue.

Subsequently we gave 10 g. guaiacol carbonate alone to the same pig (Fig. 1, day 25) and again obtained only a minute rise in the ethereal sulphate, a rise too small for any comparison with the previous guaiacol experiment. To the second pig (Fig. 2, day 18) we administered 4·4 g. *p*-iodophenol. We

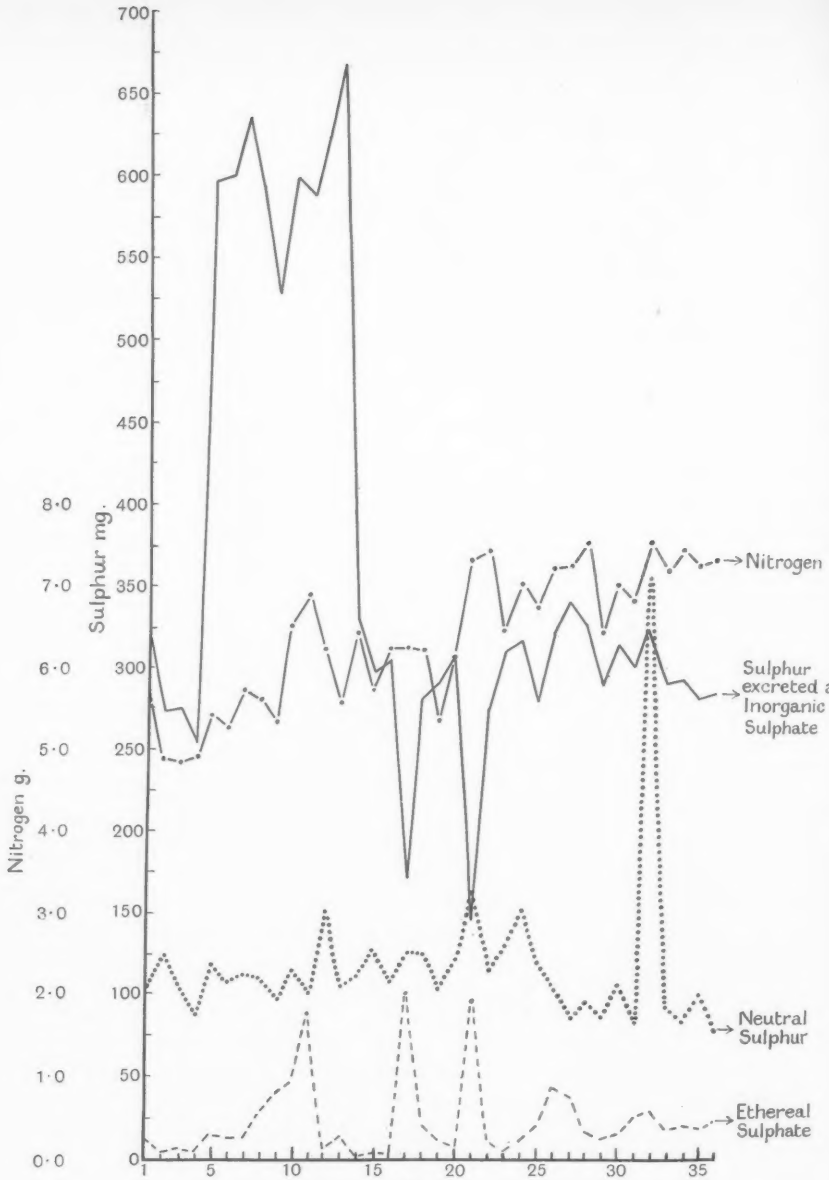

Fig. 1. "Phyllis."

Days No. 5 to 13. 4 g. Na_2SO_4, $10H_2O$ daily.
,, No. 8 and 9. 10 g. guaiacol carbonate daily.
,, No. 11. 3 g. phenol.
,, No. 17. 4·08 g. (2 × M.W./100 g.) iodobenzene.
,, No. 21. 3·14 g. (2 × M.W./100 g.) bromobenzene.
,, No. 25. 10 g. guaiacol carbonate.
,, No. 32. 3·65 g. (1 × M.W./100 g.) *p*-iodophenylmercapturic acid.

This figure may be compared directly with the figures in Papers III, IV and V of this series. The figure in Paper I is, however, drawn to a different scale.

used the iodine compound as the output of iodine in the urine afforded an indication of absorption and excretion. From the iodine figures it was clear that 76 % of the dose was excreted on the first day and 8 % on the second day. The ethereal sulphate analyses indicated an excretion of only 10 % of the dose in this form. The dogs "Vixen" and "Sophia" excreted 59 % and 53 % respectively of the corresponding chloro-compound in the form of ethereal sulphate [Coombs and Hele, 1926]. These results are summarised in Table I.

Table I. *The formation of ethereal sulphate.*

Percentage of dose of various compounds excreted as:

	Ethereal sulphate		Inorganic sulphate	
	Pig %	Dog %	Pig %	Dog %
Sodium sulphate alone	Nil	Nil	81	89
Guaiacol carbonate alone	2·5	12	Nil	Nil
Guaiacol carbonate and sodium sulphate together:				
Guaiacol carbonate	2·4	30	Nil	Nil
Sodium sulphate	Nil	66	69	22
Phenol alone	—	38	—	Nil
		57		
p-Chlorophenol alone	—	53	—	Nil
		59		
p-Iodophenol alone	10*	—	Nil	—
Phenol and sodium sulphate together:				
Phenol	8	—	Nil	—
Sodium sulphate	Nil	—	75	—

The numbers for the dog are taken from Papers I and IV of this series.

* The iodine excreted in the urine indicated that 84 % of the *p*-iodophenol was excreted.

It is clear from these experiments that ethereal sulphates are less readily formed in the pig than in the dog. We have no proof of the fate of the large balance of the absorbed phenols. The urine of course contained glycuronic acid and the increase in the reducing power of the urine suggested that the balance of the phenols was excreted combined with this substance.

In no instance was any rise in neutral sulphur observed in the urine after the administration of phenols; in this respect the pig resembles the dog.

The effect of the monohalogenbenzenes on metabolism.

We administered 4·08 g. iodobenzene to the first pig (Fig. 1, day 17) but did not obtain any rise in the neutral sulphur of the urine. On the dose-day there was a rise of 98 mg. S in the ethereal sulphate fraction and 18 mg. on the following day, representing in all only 18 % of the dose. In the dog the same dose would have yielded a rise in neutral sulphur and ethereal sulphate, corresponding respectively to 47 % and 25 % of the dose. Taking the two sulphur fractions together, 18 % in the pig should be compared with 72 % in the dog [Coombs and Hele, 1926]. On the dose-day 1125 mg. iodine (44 % of the dose) was excreted and on the following day 369 mg. (14·5 % of the dose), showing that there had been no failure in absorption. We repeated

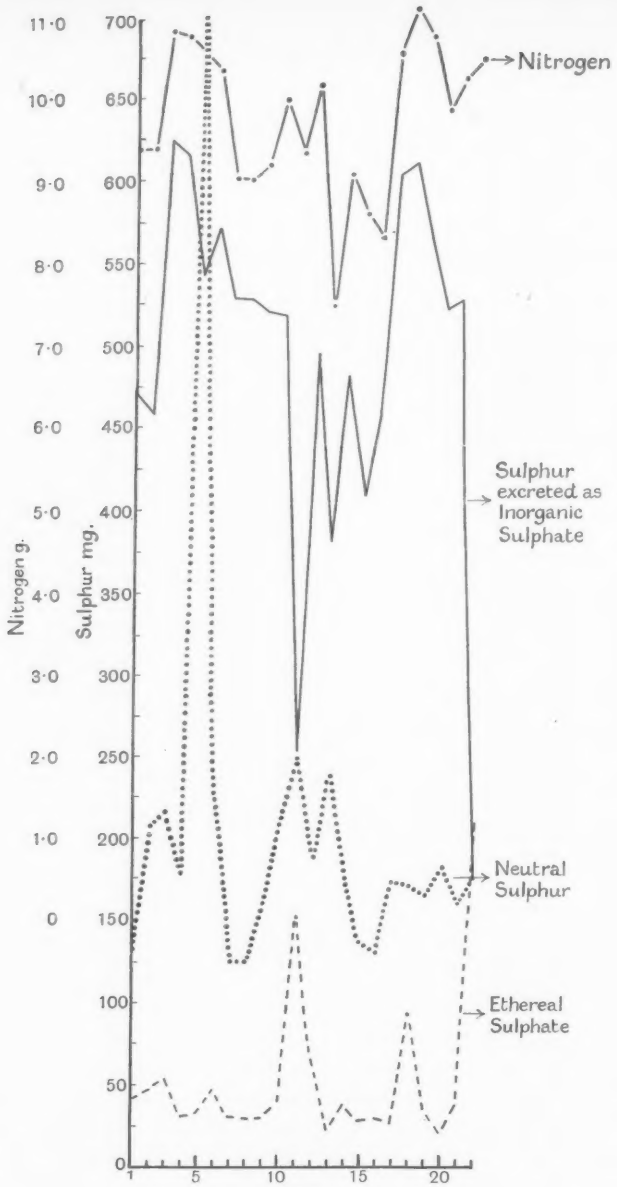

Fig. 2. "Rosamund."

Day No. 5. 6·36 g. (2 × M.W./100 g.) *p*-bromophenylmercapturic acid.
„ No. 11. 6·28 g. (4 × M.W./100 g.) bromobenzene.
„ No. 18. 4·4 g. (2 × M.W./100 g.) *p*-iodophenol.
„ No. 22. 6·12 g. (3 × M.W./100 g.) iodobenzene.

This figure may be compared directly with the figures in Papers III, IV and V of this series. The figure in Paper I is however drawn to a different scale.

the experiment with a larger dose (6·12 g.) on the second pig (Fig. 2, day 22) and obtained exactly similar results.

We administered 3·14 g. bromobenzene to the first pig (Fig. 1, day 21) and obtained a small rise in the output of neutral sulphur, equivalent to 6·7 % of the dose. This increase might be attributed to normal variations in metabolism. The rise in ethereal sulphate corresponded to 14 % of the dose. A larger dose (6·28 g.) was given to the second pig (Fig. 2, day 11). The rise in ethereal sulphate on the dose-day indicated an excretion of 10 % of the dose and on the following day 3 %. The effect on the neutral sulphur is not so easy of interpretation. The neutral sulphur had risen to 212 mg. on the previous day. The actual increase over this figure on the dose-day was only 59 mg. S (4·6 % of the dose). This was followed by a fall and a subsequent rise. We regarded these variations as extraneous in origin and without relation to the administration of the bromobenzene. In the dog the rise in neutral sulphur would have been about 600 mg. S. However, the fact that a small rise in neutral sulphur did occur in the pig after bromobenzene made us attempt the isolation of a mercapturic acid from bromobenzene and also from iodobenzene. Although the usual precautions were taken, we failed to isolate any. The results are summarised in Table II.

Table II. *The effect of the monohalogenbenzenes on metabolism.*

Percentage of dose excreted as:

	Ethereal sulphate		Neutral sulphur		Halogen	
	Pig	Dog	Pig	Dog	Pig	Dog
	%	%	%	%	%	%
Iodobenzene	18	25	Nil	47	58·5	79
	19	30	Nil	47	51	68
Bromobenzene	14	28	6·7 (?)	47·2	—	—
	13	25	4·6 (?)	62·6	—	—
Chlorobenzene	—	24·9	—	34·8	—	57·9
	—	19·2	—	35·8	—	55·2

The numbers for the dog are taken from Papers II, III and IV of this series.

The effect of the mercapturic acids on metabolism.

To the first pig (Fig. 1, day 32) we administered *p*-iodophenylmercapturic acid. The rise in the neutral sulphur output on the first day corresponded to 83 % and the iodine excretion to 76 % of the dose. There was no appreciable rise in the ethereal sulphate. The excretion of the mercapturic acid was over within 24 hours. There was no haemoglobinuria.

As a control an equivalent dose of *p*-iodophenylmercapturic acid was given to a large female dog. The pig, weighing 18 kg. received 3·65 g., the dog, weighing 11 kg., received 2·23 g. The dog's urine was free from haemoglobin for the first 24 hours following the dose. For the next 23 hours the urine contained haemoglobin but on the third day the urine was clear. On the first day the iodine estimations in the urine showed an excretion of only 9 % of the dose, on the second day 12 %. The haemoglobinuria showed exactly

the same features as those observed following the administration of the bromo- and chloro-compounds [Callow and Hele, 1927]. It is interesting to compare the rapid excretion of mercapturic acid by the pig and the slow excretion by the dog.

To the second pig (Fig. 2, day 5) 6·36 g. *p*-bromophenylmercapturic acid, or twice the equivalent dose of the *p*-iodophenylmercapturic acid used for the first pig, were given. The rise in the neutral sulphur indicated that 80 % of the dose was excreted in the first 24 hours and 6 % on the following day. None was converted to ethereal sulphate. There was no haemoglobinuria. If the dosage, g. per kg. of body-weight, is compared, this dose is nearly three times the amount which produced haemoglobinuria in the dog [Callow and Hele, 1927]. More than half of the mercapturic acid administered was recovered unchanged from the urine. The results are summarised in Table III.

Table III. *The effect of the mercapturic acids on metabolism.*

Percentage of dose excreted as:

	Ethereal sulphate		Neutral sulphur		Halogen	
	Pig %	Dog %	Pig %	Dog %	Pig %	Dog %
p-Iodophenylmercapturic acid	Nil	Nil	83	(?)	76	21
p-Bromophenylmercapturic acid	Nil	Nil	86	48	—	—
p-Chlorophenylmercapturic acid	—	Nil	—	(?)	—	36

The numbers for the dog are taken from this paper and from Paper V of this series.

Discussion.

From our results we feel justified in concluding that the metabolism of our pigs was very different from that of our dogs. The pig seems incapable of synthesising mercapturic acids. It is clear also that the pig does not synthesise ethereal sulphates with the same readiness as does the dog. In our experiments glycuronic acid was excreted and the increase in the reducing power of the urine, following the administration of phenolic compounds, suggested that the pigs detoxicated foreign substances of this sort mostly by the glycuronic acid synthesis, but there is no exact proof. It is to be noted that in our experiments with one exception (*p*-iodophenol, Fig. 2, day 18) the rise in ethereal sulphate was accompanied by a fall in inorganic sulphate. We know that the dog can synthesise ethereal sulphate directly from at least one phenol and sulphate [Hele, 1924, 1] and we have no reason to believe that the pig cannot do the same. However, we regard the conclusive proof of this in the pig as extremely difficult or impossible, because the pig does not synthesise ethereal sulphates readily.

Shiple, Muldoon and Sherwin [1924] express the view that there are two ways of detoxicating phenolic substances, the one by union with some intermediary sulphur compound of endogenous metabolism, which is oxidised to ethereal sulphate, and the other by utilising exogenous cystine to form a mercapturic acid or like substance, which may be either excreted unchanged or oxidised to ethereal sulphate wholly or in part. In the pig they obtained

a rise in neutral sulphur after the halogen-benzenes but were apparently unable to isolate a mercapturic acid from that animal [Muldoon, Shiple and Sherwin, 1923]. On the other hand, they believe that ethereal sulphates cannot be formed by the direct union of phenols with preformed sulphate, whether arising endogenously or exogenously. Their views are based on work on two animals, a dog of 15·5 kg. [Muldoon, Shiple and Sherwin, 1924] and a pig of 24 kg. [Shiple, Muldoon and Sherwin, 1924]. Their ideas are in accordance with theories put forward by Thomas and Straczewski, Zeller and Straczewski, Kapfhammer and Rhode, theories already discussed in this series [Hele, 1924, 2]. It is not clear however that the experimental evidence adduced by the American workers will bear none other than the interpretation put upon it by the authors.

In the work on the dog [Muldoon, Shiple and Sherwin, 1924, p. 679] the only suggestion of a controversial kind is the theory put forward that ethereal sulphate is solely formed from endogenous sulphur. The experimental basis for this theory is the fall in output of ethereal sulphate when cystine is given to a dog receiving a daily dose of bromobenzene. There does not seem, however, conclusive proof that such a fall occurs under these circumstances. The authors consider mercapturic acid to be derived from the union of bromobenzene with exogenous sulphur and ethereal sulphate from the union of bromobenzene with endogenous sulphur. Any increase therefore of exogenous sulphur would tend to upset the balance, increasing the mercapturic acid at the expense of the ethereal sulphate. They assume as others have done that the intermediary in either case is a phenol. We have however shown [Coombs and Hele, 1926] that the phenols are not intermediaries in the synthesis of mercapturic acids. When the series of reactions

Ethereal sulphate ← *p*-bromophenol ← bromobenzene → mercapturic acid
(Union with sulphate) (Union with cysteine)

is considered, it is clear that the decrease in ethereal sulphate, if it really does occur, must be due to an increased formation of mercapturic acid as the concentration of available cysteine is raised. There is no necessity to distinguish between endogenous and exogenous metabolism. In the second paper of this series [Hele, 1924, 2] stress was laid on the uniformity of the response of dogs to the halogen-benzenes, but some evidence, not very conclusive, was put forward in that paper to prove that the balance could be upset, if dosage with sulphate or with cystine was continued for more than one day.

The experimental work on the pig summarised in the first table of the American authors (p. 61) is not antagonistic to our experimental results and our views except in one particular. They found an increase in ethereal sulphate following the administration of *p*-bromophenylmercapturic acid. Perhaps it may be allowed that animals of the same species may vary in their power to carry out certain oxidations. We find it, however, very difficult from structural considerations to conceive of any oxidation of a mercapturic acid to ethereal sulphate without the fission of the molecule and the liberation of

a phenol, which might then be detoxicated by direct union with sulphate. This suggestion has already been mooted in the second paper of this series [Hele, 1924, 2]. An oxidation of a mercapturic acid to a sulphonic acid, the sulphur of which would still appear in the neutral sulphur fraction, is much more likely to occur [König, 1892]. In the rest of the table evidence is collected, which shows that the oral administration of cystine assists in the formation of ethereal sulphate from phenols, while sodium sulphate does not. We would expect cystine to be the more efficacious, because it is excreted more slowly, being in an unoxidised form, and because very likely it is oxidised at the seat of ethereal sulphate synthesis, thus producing sulphate where it is most required [Hele, 1924, 1]. For reasons already given we feel it would be very difficult to obtain conclusive proof of the formation of ethereal sulphate in the pig from preformed sulphate, even if the nitrogen-free diet, utilised by the American workers, were employed. Contrary to their findings we obtained generally a fall in inorganic sulphate when the ethereal sulphate rose. The pig behaves in this respect like the dog.

In the second table (p. 62) of the American work on the pig there are several findings contradictory to our theories. Bromobenzene caused a rise in neutral sulphur, and added cystine caused a further rise with a decrease in the output of ethereal sulphate. The effects of *p*-chlorophenol and cystine were similar. The fall in ethereal sulphate, which is not very great, may have been accidental and part of the neutral sulphur may be attributed to failure in oxidation of the cystine apart from any effect of the derivatives of benzene. This is certainly true in the *p*-chlorophenol—cystine experiments. The rise in neutral sulphur is about 400 mg. S. The dose (0·6 g.) of *p*-chlorophenol given could not theoretically have given rise to more than 150 mg. S. In this table there is also further evidence of the oxidation of *p*-bromophenylmercapturic acid.

In the American work on the pig there are only two findings which are seriously antagonistic to our theories; the rise in neutral sulphur after bromobenzene and the similar rise after *p*-chlorophenol. We believe that phenols bear no relation to the formation of mercapturic acids [Coombs and Hele, 1926] and even if these findings of the American workers are incontestable, it does not follow that such a rise in neutral sulphur indicates the formation of a mercapturic acid. It might be due to a sulphonic acid.

There are two defects in the work of the American authors, which are not unimportant. The variations in the output of nitrogen are considerable. These variations cannot always be attributed to the nature of the substances administered. Such variations indicate variations also in the metabolism of tissue sulphur, variations which would affect the neutral sulphur of the urine. It is true that these variations are most marked in the first "pig" table, where they have but little bearing on the authors' argument and it is probable that such variations are bound to occur when a nitrogen-free diet is employed, or at any rate are more fully exposed to view when the nitrogen excretion is

low. The second experiment described in this paper is open to the same criticism in a lesser degree. We have however taken into consideration these variations in nitrogen output and we do not think that our conclusions are inadmissible.

The second defect in the American work is avoidable. We have always found it necessary to allow a considerable interval of time to elapse between each stage of an experiment, in order to make certain that the metabolism has again reached the normal. It is true that in our first "pig" experiment we administered phenol almost immediately after guaiacol carbonate. In this particular experiment however we were testing merely the general effect of phenolic substances. The administration of guaiacol and phenol constituted one experiment. We have made an approximate division in the output of ethereal sulphate between these substances in the table as an indication of a general thesis. The American workers, however, were carrying out many different tests, and in our opinion they did not leave sufficient intervals of time between each stage of their enquiry. It is not always clear to what cause to attribute a particular effect.

In our experiments we have found that mercapturic acids produce haemoglobinuria in the dog, an animal capable of synthesising these compounds. On the other hand, we have found that the pig cannot synthesise mercapturic acids and that mercapturic acids in much larger doses per kg. of body-weight have no effect on the pig. The American workers also do not describe any toxic effects following the administration of mercapturic acids to the pig and the same seems true for the rabbit [Rose, Shiple and Sherwin, 1924]. Perhaps, too, the rabbit is incapable of synthesising mercapturic acids [Muldoon, Shiple and Sherwin, 1923]. This difference indeed seems remarkable, although it is possible that mercapturic acids in massive doses might cause haemoglobinuria even in the pig. The rapid excretion of mercapturic acids by the pig may be attributed to the fact that the kidney of the pig is not in any way blocked by red cell debris, the result of haemolysis.

Summary.

1. The pig, unlike the dog, does not readily synthesise ethereal sulphate. For this reason it would be extremely difficult, if not impossible, to obtain conclusive evidence of the direct formation of ethereal sulphate from phenolic compounds and preformed sulphate. On the other hand, there is no evidence that the pig does not carry out this direct synthesis in the same manner as the dog.

2. The pig unlike the dog is probably unable to synthesise mercapturic acids.

3. The pig like the dog cannot convert mercapturic acids to ethereal sulphates.

4. Mercapturic acids in doses three times the toxic dose for the dog per kg. of body-weight do not produce haemoglobinuria in the pig.

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LXXXVI. STUDIES IN THE SULPHUR METABOLISM OF THE DOG.

VII. THE EFFECT OF FLUOROBENZENE ON SULPHUR METABOLISM.

By HERBERT ISAAC COOMBS.

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(*Received May 2nd, 1927.*)

In a former communication of this series [Coombs and Hele, 1926] it was shown that very small differences in chemical constitution very much alter the metabolism of the halogen-benzenes (chloro-, bromo- and iodo-benzenes). These latter bodies all give rise to a mercapturic acid in the urine of dogs after they have been ingested. They are, however, the only compounds which have been definitely proved to cause the synthesis—the substitution of even such a group as methoxyl in the ring causing the metabolism to take a different course. Evidence has been obtained, however, that the *o*- and *m*-dichlorobenzenes and benzene itself probably also give rise to mercapturic acids [Callow and Hele, 1926].

Perhaps the most likely compound which might be expected to form a mercapturic acid is the remaining halogen-benzene, fluorobenzene. This compound fits exactly into the series with the other halogen-benzenes in all its chemical and physical properties, and there would therefore seem no reason why it should not also have a similar effect on sulphur metabolism. To test this point a quantity of fluorobenzene was made and administered to dogs with the results shown in the figures. Benzene itself was also administered with the result shown.

Experimental.

About 150 g. fluorobenzene were prepared by the method of Holleman [1904]. The method gave a very pure product but not in very good yield; the only difficulty that was encountered was the solvent action of the mercury of the mercury seal on the solder of the reaction vessel, but this was easily remedied by the substitution of a seal turned from one piece of copper.

The diet of the dog "Vixen" has been given in a former paper of this series and that of "Pansy" was as follows[1]:

		T.N.	T.S.	Calories
"Pansy," 8·6 kg.:		g.	g.	K
Meat	80 g.	2·73	0·181	106
Sugar	70 g.	—	—	286
Margarin	20 g.	—	—	156
Milk	160 cc.	0·83	0·074	115
		3·56	0·255	663

[1] This diet is similar to that given for "Pansy" in Paper V, but differs in sugar content.

It will be seen from the figures that there is a small but distinct rise in the neutral sulphur output following the administration of the fluorobenzene —amounting to about 8 to 11 % of the dose. This rise is small compared to the rise after the other halogen compounds where it is about 47 % [Coombs and Hele, 1926], and is more in accord with that caused by benzene. It must be remembered, however, that there is a great difference in the boiling points —fluorobenzene boiling at 85° whereas chlorobenzene boils at 132°. The disparity in the yield from these two compounds must be attributed, in part at least, to this volatility, because shortly after the fluorobenzene has been ingested it can easily be detected by its odour in the breath of the animal.

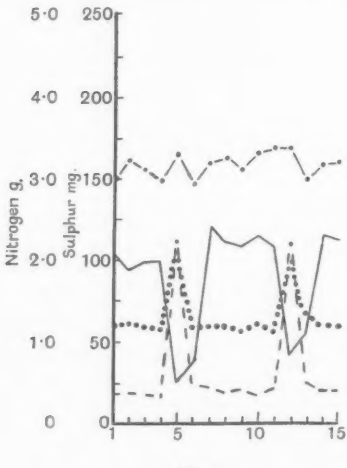

Fig. 1.

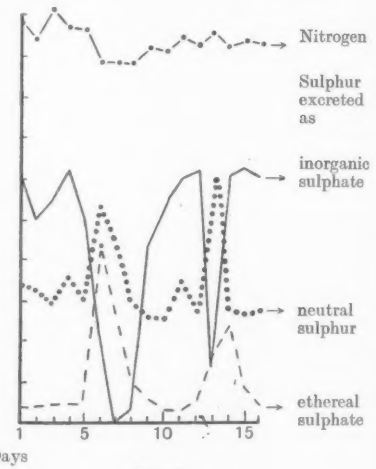

Fig. 2.

Fig. 1. "Pansy." Day No. 5. 2 × M.W./100 g. fluorobenzene } by mouth.
„ No. 12. 2 × M.W./100 g. fluorobenzene }

Fig. 2. "Vixen." Day No. 6. 2 × M.W./100 g. fluorobenzene } subcutaneously.
„ No. 13. 2 × M.W./100 g. benzene }

The author's best thanks are due to Dr T. S. Hele for his interest in this work, and the author also wishes to acknowledge gratefully grants from the Royal Society and the Medical Research Council.

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LXXXVII. THE REDUCTION OF HAEMATIN AND METHAEMOGLOBIN.

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(*Received April 26th, 1927.*)

The question of the oxygen content of methaemoglobin relative to haemoglobin has long interested physiologists. The recent work of Keilin [1926] has brought haemochromogen into prominence as a type of important biological substances. The great ease with which haematin is reduced in comparison with protoporphyrin has led to the assumption that in the reduction of the former to reduced haematin the essential change is that of the valency of the iron. This would involve one equivalent of hydrogen per atom of iron. Küster [1910] suggested that methaemoglobin was a compound of haematin in which the iron was in the ferric state. In oxyhaemoglobin, haemoglobin, haemochromogen and reduced haematin the iron might therefore be held to be in the ferrous state. If this is the case, the term "oxygen content" of methaemoglobin and haematin has little meaning, as is pointed out by Conant and Scott [1926]. Determinations of the equivalents of hydrogen necessary for the reduction of haematin have failed to confirm this view. The data and conclusions of many workers are summarised by Nicloux and Roche [1926]. As a result of their own experiments these workers concluded that the reduction of methaemoglobin required two equivalents of hydrogen. Later, Roche [1926] contended that haematin needs four equivalents for its reduction. Their work has been discussed by Conant and Scott [1926].

Conant [1923] had previously titrated electrometrically both methaemoglobin and haematin and found one equivalent of hydrogen necessary for the former but two for the latter substance. He, however, considered the method unsuitable for haematin.

Haurowitz [1927] had reduced haematin with hydrazine hydrate and by measuring the nitrogen evolved found one equivalent for the reduction. It must however be taken for granted that the volume of nitrogen measured corresponds with the whole of the hydrogen used in reducing the pigment, for which the available evidence is satisfactory. For the reduction of haematin to reduced haematin, hydrazine hydrate is unsuitable because in itself it is a nitrogen compound capable of yielding a haemochromogen [Anson and Mirsky, 1925]. Haurowitz does not seem to have realised any difference between reduced haematin and haemochromogen.

Hill and Holden [1926] prepared methaemoglobin by direct combination of haematin and globin. In view of the discrepancies in the above results it was decided to attempt to titrate directly with a reducing agent solutions of methaemoglobin, of haemin in alkali, and of haemin in alkali in the presence of pyridine.

The essential requirement of such a research was that it should be simple and direct both in theory and technique.

The choice of a suitable reducing agent was the first consideration. From the limited number of available reducers ferrous tartrate in alkaline solution was selected. In practice, standard ferrous sulphate solution was added to an alkaline solution of the pigment containing sodium tartrate. By this means errors due to atmospheric oxidation of the standard reducing agent itself were reduced to a negligible amount. The only state to which a ferrous salt is readily oxidised is that of a ferric salt. The reduction potential of the simpler ferrous compounds increases greatly on passing from an acid to an alkaline reaction. Excess of ferric tartrate (the product of its oxidation) was found to be without appreciable influence. No additional standardisation is necessary if pure ferrous ammonium sulphate is used. The possibility of the reaction stopping at the formation of ferrosoferric compounds is negatived by the indifference of excess of ferric tartrate.

As ordinarily prepared, methaemoglobin contains undesirable amounts of ferricyanide which are not readily removable by dialysis. Nicloux's method gives a 60 % conversion of oxyhaemoglobin to methaemoglobin, but such a solution was unsuitable for these experiments. The preparation of a sufficient quantity of pure globin was too tedious owing to its high equivalent weight. Accordingly advantage was taken of the fact that at an acid reaction at which oxyhaemoglobin is still stable ferric tartrate oxidises it completely to methaemoglobin. If such a solution be immediately made alkaline the ferrous salt formed reduces some of the methaemoglobin. It was found possible to remove the ferrous salt and the bulk of the ferric salt by suitable treatment without such reduction occurring.

In the case of haematin, since both reduced haematin and pyridine haemochromogen are insoluble, they can be separated from the soluble haematin by centrifuging. The end-point can thus be determined.

In the case of methaemoglobin the oxyhaemoglobin produced by a known amount of reducer followed by oxygen was estimated by determining the oxygen capacity of the solution.

The preparation of methaemoglobin.

1 litre of fresh defibrinated ox blood was centrifuged and the corpuscles washed three times with 0·9 % sodium chloride solution. To 500 cc. of corpuscles so obtained were added 4·5 l. of water, 500 cc. 25 % sodium tartrate (Rochelle salt is not suitable) and 250 cc. 10 % acetic acid by volume. After

thorough mixing, 60 g. sodium tartrate and 40 g. iron ammonium alum dissolved in 500 cc. of cold water were slowly added with stirring. Bubbles were given off in the liquid and the methaemoglobin spectrum rapidly replaced that of oxyhaemoglobin. After standing the turbid liquid was filtered from the stroma, etc., the clear filtrate was diluted with twice its volume of water and allowed to stand overnight at 0°. The methaemoglobin was practically completely precipitated as a granular precipitate. This precipitate contained ferric tartrate and was completely insoluble in distilled water. It was soluble in very dilute alkalis yielding first a clear neutral solution of methaemoglobin, from which the iron could be dialysed. That the precipitating agent was ferric and not ferrous tartrate could be shown as follows. On suspending the washed precipitate in distilled water and adding a small amount of sodium hydrosulphite, as reduction proceeded the precipitate dissolved, finally yielding a perfectly clear solution of haemoglobin from which oxyhaemoglobin was obtained. The granular precipitate of methaemoglobin was washed with 2 l. of distilled water containing 2 cc. 10 % acetic acid and 20 cc. 25 % sodium tartrate. After expressing the superfluous liquid the precipitate was ground into a cream with water and dissolved with the minimum amount of 10 % sodium carbonate, added slowly from a burette, about 100 cc. being required. To the 620 cc. of solution so obtained were added 5 cc. of 25 % sodium tartrate and enough saturated ammonium sulphate to make a total volume of 1 l. The solution was then filtered. To the filtrate 1·5 l. saturated ammonium sulphate were added and, after standing overnight at 0°, the precipitate of methaemoglobin was filtered off. The iron remained in the filtrate. After washing with saturated ammonium sulphate the precipitate was pressed as dry as possible between filter papers and dissolved in half its weight of distilled water. This strong methaemoglobin solution was used directly for the experiments. After complete reduction of a measured sample with ferrous tartrate its oxygen capacity was determined by Barcroft's method. The tint of the oxyhaemoglobin of this sample was compared at known dilution with diluted defibrinated blood of known oxygen capacity. They were found to agree, showing that all the haematin present was in the form of methaemoglobin. Hence the precipitation of methaemoglobin by ferric tartrate under the conditions of the experiment does not alter the essential properties of the conjugated protein. When freed from the iron tartrate the oxyhaemoglobin finally obtained resembles in solubility, oxygen capacity, and colour the original oxyhaemoglobin.

The reduction of haematin.

The experiments were conducted in small centrifuge tubes about 62 × 18 mm. The haematin solution was made by dissolving a known weight of about 0·3 g. in 25 cc. of dilute alkali. It was used immediately. Recrystallised ferrous ammonium sulphate was dissolved in water containing a trace of sulphuric acid to give a 0·02 *N* solution. A suitable amount of an alkaline tartrate

solution was used. A layer of toluene prevented reoxidation. To a series of four tubes were added equal amounts of haematin, alkaline tartrate solution and toluene, and an amount of water to make the total volume after addition of the reducer up to 8 cc. Four different volumes of the ferrous sulphate solution were added to the different tubes. They were stirred gently and immediately centrifuged after cautious removal of the stirrers. After 5 minutes the tubes were removed from the centrifuge and observed. In Exp. 1 they were observed in sunlight without centrifuging. The details of two typical experiments are recorded and the results for a series given in Table I.

Table I.

For 2 cc. haematin titrated with standard ferrous iron solution as reducer.

Remarks	cc. reducer added to effect reduction	Observed correction in cc. of reducer for dissolved oxygen	cc. reducer used in reducing haematin	Theoretical cc. reducer for 1 equivalent	Equivalent
Pyridine haemochromogen in sodium carbonate	2·40	0·35	2·05	2·31	0·89
Same, with 1·5 cc. *N*/5 ferric iron	2·30	0·35	1·95	2·31	0·84
Reduced haematin in sodium hydroxide	2·10	0·30	1·80	1·81	0·99
Pyridine haemochromogen in sodium hydroxide	2·30	0·35	1·95	1·95	1·00
Pyridine haemochromogen in sodium hydroxide	2·20	0·35	1·85	1·95	0·95

Exp. 1. The alkaline tartrate is a mixture of 20 cc. 10 % Na_2CO_3 + 10 cc. 25 % sodium tartrate. Ferrous ammonium sulphate, 0·4015 g. in 50 cc. Haemin 0·3329 g. in 25 cc. containing 0·2 cc. purified pyridine. Each tube contained 2 cc. haematin solution + 2 cc. alkaline tartrate + 1 cc. toluene and water to make 9 cc. total volume.

Tube	Ferrous sulphate cc.	Observation
1	2·3	Nearly reduced
2	2·5	Reduced
3	2·7	,,
4	4·0	,,
Zero correction for dissolved oxygen using very dilute haematin.		
1	0·1	Not reduced
2	0·2	,,
3	0·3	,,
4	0·4	Reduced

Result. 2 cc. of 0·0237 *N* haematin required 2·40–0·35 cc. of 0·0205 *N* iron.

Exp. 2. The alkaline tartrate is a mixture of 1 cc. 25 % sodium tartrate, 1 cc. 40 % sodium hydroxide and 1 cc. of water. Ferrous ammonium sulphate; 0·8073 g. in 100 cc. Haemin; 0·6054 g. to 50 cc. Each tube contained 2 cc. haematin solution + 2 cc. alkaline tartrate + 2 cc. toluene + water to make 9 cc. total volume.

Tube	Ferrous sulphate cc.	Observation
1	1·8	Not reduced
2	2·0	,,
3	2·2	Reduced
4	2·5	,,
Zero correction using very dilute haematin.		
1	0·1	Not reduced
2	0·2	,,
3	0·3	Just reduced
4	0·4	Reduced.

Result. 2 cc. of 0·0186 *N* haematin required 2·1–0·30 cc. 0·0206 *N* iron.

The reduction of methaemoglobin.

5 cc. of a strong solution of methaemoglobin prepared as described above were measured into a test-tube. To this was added 0·7 cc. of a solution prepared by mixing 50 cc. 25 % tartrate, 36 cc. water and 14 cc. of strong ammonia solution (Sp. Gr. 0·880). A measured quantity of water was added so that, with the volume of the reducing agent to be added subsequently, the total volume of liquid would be 8·5 cc. A stirrer was then placed in the tube and a layer of liquid paraffin 1 cm. deep carefully poured on. The ferrous ammonium sulphate solution (containing 0·2023 g. of the salt in 25 cc. of water) was run in from a burette with a long drawn-out jet reaching nearly to the bottom of the tube. The stirring was continued for 1 minute after the reducer had been added and the mixture allowed to stand 15 minutes. The solution of haemoglobin was then drawn up in a pipette from beneath the paraffin and transferred to a conical flask where it was gently shaken for 15 minutes with oxygen from a cylinder. 3 cc. of the resulting solution of oxyhaemoglobin were measured out into the bulbs of two Barcroft differential gas apparatus. The oxygen capacity was then directly determined by the addition of ferricyanide. The results of the experiments are set forth in Table II.

Table II. *Reduction of methaemoglobin as measured by oxygen capacity with standard ferrous iron solution.*

cc. reducer added	Observed oxygen capacity cc.	Calculated oxygen capacity for 1 equivalent cc.	Calculated oxygen capacity for 2 equivalents cc.	Observed equivalent
2·35	838 848	948	474	1·11 1·10
2·30	806 788	920	460	1·12 1·15
2·30	781 751	920	460	1·16 1·20

A similar series of experiments was now made using a Haldane evolution apparatus. Different amounts of reducer were added and the corresponding oxygen capacities determined by adding ferricyanide. The zero correction for

dissolved oxygen was determined as in the case of haematin, using a very weak solution of methaemoglobin. The results of this series are shown in Fig. 1. The ordinates represent the oxygen capacity in cc. in terms of 5 cc. of the original methaemoglobin solution, the abscissae represent cc. of a standard iron solution (0·02 *N*) added. The dotted curves represent the theoretical oxygen capacities for the reduction involving one and two equivalents of iron per molecule of methaemoglobin.

Discussion of results.

The high equivalent weights of haemin (651) and of methaemoglobin (16,700) militate against the experimental accuracy with which the addition to either of one equivalent of hydrogen can be determined. In addition, both substances are known to undergo certain changes in solution. The reaction between reducing agents and such decomposition products is as yet obscure. Such decomposition might be expected to affect the two methods of estimation employed in opposite directions decreasing the amount of oxygen obtainable

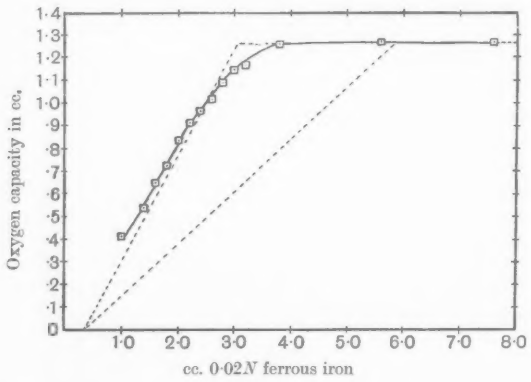

Fig. 1.

from the conjugated protein after treatment with a given amount of reducer and decreasing the amount of reducer required for a known volume of haematin solution. The mean value for the case of haemochromogen and reduced haematin is 93 % of the theoretical value for one equivalent of hydrogen. Of the two samples of haemin used the more recently prepared one gave the values most nearly approaching to the theoretical value. The determinations of the resulting oxygen capacity on reduction of a single specimen of methaemoglobin showed a progressive decrease from day to day. The mean value of four determinations carried out within 2 days after the preparation of the methaemoglobin gave a ratio of equivalents of hydrogen/molecular oxygen of 1·12. The effect of reducible impurities in the methaemoglobin is clearly shown by the curve. If the methaemoglobin were perfectly pure, a sharp break in the

curve would be expected to occur when the theoretical amount of reducer had been added. The curve, however, tails off gradually to a constant oxygen capacity corresponding to complete reduction. The methaemoglobin, however, is completely reduced long before two equivalents of reducer have been added, and for smaller amounts of reducer closely follows the slope of the theoretical curve for one equivalent.

It would seem probable that the change of methaemoglobin to haemoglobin or of haematin to either reduced haematin or haemochromogen is one and the same, namely, the addition of one equivalent of hydrogen per atom of iron. The protein would appear to play no essential part in this reduction and does not react with the reducing agent. Methaemoglobin is a compound of haematin and globin while haemoglobin is a compound of reduced haematin and globin. Küster's hypothesis [1910] is thus experimentally confirmed.

SUMMARY.

1. Methaemoglobin has been prepared by the use of ferric tartrate instead of potassium ferricyanide.

2. The protein can be completely precipitated by ferric tartrate without denaturation.

3. The reduction of methaemoglobin to haemoglobin requires one equivalent of hydrogen per atom of iron.

4. The reduction of haematin to reduced haematin or to pyridine haemochromogen requires one equivalent of hydrogen per atom of iron.

The authors desire to record their gratitude to Sir F. G. Hopkins for his advice and encouragement. One of them (R. H.) is indebted to the Medica Research Council for a grant.

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LXXXVIII. THE PHYSIOLOGICAL RÔLE OF VITAMIN B. PART III.

STUDY OF VITAMIN B DEFICIENCY IN PIGEONS[1].

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(*Received March 23rd, 1927.*)

The recognition that there exists a close relationship between the consumption of food and the supply of vitamin B in the diet has greatly advanced attempts to determine the physiological rôle of this substance. It has, in the first place, provided a plausible explanation of many of the numerous divergencies of opinion that are recorded in the literature, whilst secondly it has, we believe, indicated the right line along which future research in this field should proceed.

It is obvious from a survey of the literature that the great majority of investigators have disregarded entirely or failed to appreciate fully the significance of the voluntary abstention from food that animals deprived of vitamin B usually exhibit. A few have borne this in mind, notably Funk [1924, p. 196].

Object of the experiments.

In view of the conclusions reached by Drummond and Marrian [1926] concerning the part played by inanition in vitamin B deficiency in rats it seemed imperative that similar experiments should be carried out on pigeons. It was therefore planned to study the fate of pigeons fed on dietaries lacking vitamin B, but under conditions where the effects of inanition could be separated from those due more directly to the deficiency.

It is obvious that the experimental study of vitamin deficiency can give clear-cut results only when the vitamin under investigation is the sole limiting factor of a diet complete with regard to all other components and when all factors liable to affect the experiment are checked by the use of proper controls. In the present paper a new method of studying vitamin B deficiency is described, as well as the results obtained on pigeons with the aid of this method, which it is believed provides a control covering the disturbing effects of inanition.

Not less than six experimental animals are placed on the deficient diet. For each of them a control of the same weight and if possible the same age and sex is selected. The control animals are given the same amounts of the diet

[1] Recent work has clearly demonstrated the existence of at least two substances of the vitamin type in the yeast extracts employed by us. The name vitamin B used throughout this paper covers both these factors and possibly others yet undiscovered.

as are eaten voluntarily by the corresponding animals fed on the deficient diet, but in addition receive a liberal supply of the missing factor. Both lots are thus placed under identical conditions, except for the vitamin factor. It is therefore reasonable to assume that differences of behaviour exhibited by the deficient group as compared with the controls are attributable to a lack of the vitamin[1].

Experimental.

Pigeons were used as experimental animals, and were divided into four groups of eight birds, each kept in a separate cage. The individual initial weights (g.) of the pigeons are given in Table I.

Table I.

Lot I		Lot II		Lot III		Lot IV	
Pigeon	Weight	Pigeon	Weight	Pigeon	Weight	Pigeon	Weight
I *a*	414	II *a*	423	III *a*	337	IV *a*	353
I *b*	445	II *b*	443	III *b*	367	IV *b*	357
I *c*	445	II *c*	428	III *c*	398	IV *c*	400
I *d*	372	II *d*	380	III *d*	503	IV *d*	513
I *e*	457	II *e*	454	III *e*	443	IV *e*	447
I *f*	425	II *f*	433	III *f*	387	IV *f*	383
I *g*	395	II *g*	405	III *g*	411	IV *g*	410
I *h*	383	II *h*	355	III *h*	497	IV *h*	465
Average	417		415		418		416

Lot I received an artificial ration deficient in vitamin B. Each bird in Lot II received by the mouth the same quantity of food that the corresponding animal in Lot I had eaten in the previous 24 hours *plus* 1 g. of yeast extract (marmite). Lots III and IV were daily fed forcibly with amounts of the deficient ration calculated to maintain their weight if assimilated, Lot IV receiving in addition 1 g. of yeast extract every day.

Diet. A modified artificial diet of the type used by Randoin and Simonnet [1924, 1] was used. Polished rice, although much simpler to use, is by no means an adequate diet for the pigeon [McCollum and Davies, 1915; McCarrison, 1921], and in our opinion is unsatisfactory for use in such studies as these.

The ration had the following composition:

Rice starch	66 %	Butter fat	4 %
Caseinogen purified ...	16 %	Filter-paper pulp	2 %
Agar-agar	8 %	Salt-mixture (McCollum) ...	4 %

The foodstuffs were mixed with a sufficient quantity of water (4·5 litres for 6 kg. of the diet) to a stiff dough, the agar being added last, and placed in flat trays in a gas oven for 1–2 hours at 130°. The resulting loaves were cut in a bread-cutting machine into slices and then into cubes the size of small peas, and finally dried at 50–60°. The diet used for forcible feeding was not cut up, but the loaves were ground to a coarse powder after drying. The diet, when offered for voluntary consumption (Lot I), was placed in glass pots covered with a tin lid having a small hole in the middle permitting the pigeon

[1] After sending this paper to Press, we found that Gulick [1922] has described a similar technique in a short note published in the *Proceedings of the American Physiological Society*.

to pick up the diet without scattering. The quantities offered were always in excess of the daily voluntary intake and were replenished daily. For artificial feeding the powdered diet was mixed with a sufficient amount of water and placed by means of a glass syringe and a piece of rubber tubing in the pigeon's crop. The control lots (II and IV) were also given daily by means of a small funnel and a piece of rubber tubing 2·5 cc. of yeast extract solution (corresponding to 1 g. of marmite). All the birds were given once a fortnight five drops of cod-liver oil. The results obtained with Lots I and II, and III and IV will be dealt with separately.

Lots I and II.

Every day the birds were weighed and determinations of the food consumed by Lot I made. Each bird in Lot II received the same amount of food as consumed by the corresponding bird in Lot I during the previous 24 hours, together with the supplement of 1 g. of yeast extract in solution.

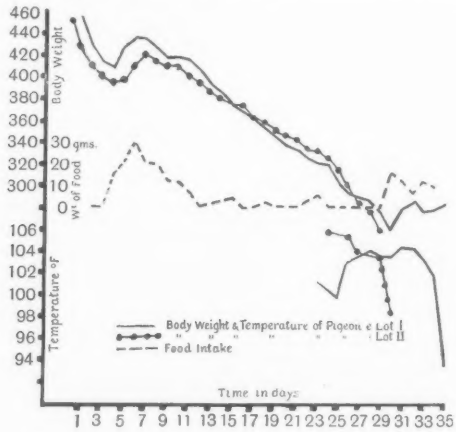

Fig. 1.

In Figs. 1, 2 and 3 are shown three typical records of body weights, food consumptions and, in the latter stages of the experiment, of body temperatures of birds in Lot I and their controls in Lot II. Fig. 4 shows the average curves for all birds in Lots I and II; this series covers a period of 28 days up to the death of pigeon *d* in Lot II. The average daily loss was 5·5 g. for Lot I and 5·0 g. for Lot II. The average daily food intake was 6·1 g. The average daily loss in complete starvation is about 9·3 g. (McCarrison). A general survey of the body weight and food intake curves demonstrates that the initial loss of weight is associated with insufficient food intake. In fact, during the first days of the experiment the pigeons, unaccustomed to be confined in individual cages, have no appetite. After a few days, however, they feed well and consume 20–30 g. of the diet, with corresponding rise in weight curves.

Later, lack of appetite becomes manifest in those deprived of vitamin B and from that time these pigeons are in a state of partial or even total starvation. In the beginning of the fourth week they sometimes regain partially their appetite and quantities of 10–15 g. of the diet may be voluntarily ingested. A possible explanation of this phenomenon will be discussed later.

Fig. 2

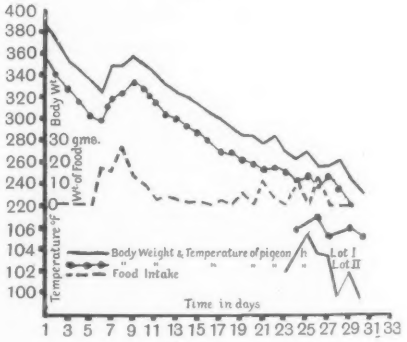

Fig. 3.

The remarkable correspondence between the curves of Lots I and II and particularly between the averages in Fig. 4 demonstrates in a striking manner the part played by inanition as a consequence of vitamin B deficiency.

Attitude of the pigeons.

The birds of Lot I, at first very active, soon became apathetic, and spent most of the time sitting motionless on the perches with eyes shut and feathers ruffled. The controls behaved quite similarly, which suggests that these symptoms are most probably due to starvation. In the later stages a period of rest-

lessness was observed and the pigeons, apparently searching for a satisfying food, scattered a great part of the diet without, however, eating appreciably. Later, as already mentioned, the food intake sometimes increased, and there was less scattering. Nervous symptoms generally followed immediately afterwards.

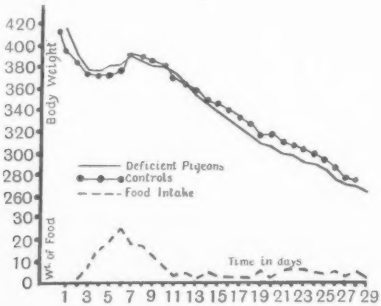

Fig. 4. Average weight and food intake of deficient and control pigeons.

Faeces.

Changes of consistence and colour of faeces of pigeons on vitamin B-deficient diets have often been described [McCarrison, 1921; Funk, 1921–25; Collazo and Funk, 1924]; the last-named authors have even devised a rough method of determining the vitamin B content of a diet based on the presence or absence of the bright green colour of the faeces; it is said that the green colour disappears in the course of 24 hours after administration of the vitamin. This finding could not be confirmed by the present work, as far as a direct relationship between vitamin B and the consistency and colour of the faeces is concerned. Emerald green coloured liquid faeces were observed as frequently in Lot II as in Lot I and their appearance seemed obviously closely related to insufficient food intake, bacterial infection playing also possibly a rôle, as McCarrison has suggested. On the other hand, faeces of absolutely normal colour and consistency have always been passed by pigeons of Lot I, even in the last stage of the experiment, following the voluntary ingestion of larger quantities of the deficient diet. Green faeces were very rare in the forcibly fed group, appearing only after rejection of food by vomiting. The presence of large amounts of undigested starch in the faeces has been described in vitamin B deficiency by McCarrison [1921] and others. However, Braddon and Cooper [1914] point out that chickens fed one-tenth of their weight of polished rice and developing acute symptoms in the course of 10 days digested 93–98 % of the starch. Similar observations were reported by Eijkman and Hoogenhuyze [1916]. Randoin and Simonnet [1924, 2] consider that pigeons deprived of vitamin B are unable to digest starch. They find, however, also large quantities of starch—more than 60 % of dry weight—in the faeces of

normal pigeons fed on a complete artificial diet. This will be discussed more fully later, and only the results of the analysis of faeces of four lots of pigeons will be given here. The faeces after drying to constant weight were hydrolysed with hydrochloric acid for 5 hours, and the reducing substances determined by Bertrand's method and expressed as "starch" by multiplying the result by the factor 0·9. The faeces were collected between the third and fourth week of the experiment.

Table II.

Pigeons on deficient diets		Pigeons on complete diets	
Lot	% of starch	Lot	% of starch
Lot I (mixed sample)	18·5	Lot II (mixed sample)	18·1
Lot III* (one pigeon)	17·0	Lot IV (mixed sample)	19·6
Lot I† (two pigeons)	20·9	—	—
Average	18·8		18·8

* It was difficult to collect samples of faeces from this group owing to contamination with vomitus.

† Taken in the last stage of the experiment.

No difference in the digestion of starch can be traced from these figures, which are actually much lower than those recorded by Randoin and Simonnet [1924, 1]. It must be remembered that agar-agar yields reducing sugars on acid hydrolysis.

Nervous symptoms.

Five out of eight pigeons of Lot I developed acute symptoms: Nos. I *a*, I *b*, I *d*, I *e* and I *h* in the course of 26, 32, 25, 25, and 30 days respectively. Pigeon I *b* had emprosthotonos, the remaining four typical opisthotonos associated with convulsions and "cartwheel" turning. I *a* and I *h* had leg weakness on the day previous to the outbreak of the acute symptoms, in the others the onset was more sudden. Of the three remaining pigeons, one, No. I *c*, received on the 33rd day of the experiment an injection of three units of insulin and was found dead on the following morning. I *f* and I *g* were killed on the 36th day of the experiment for histological examination, without having developed up to that time any acute symptoms. Pigeon I *h* was killed during the convulsions.

Spontaneous cures.

Pigeons I *a*, I *b*, I *d* and I *e* recovered without being treated, after unmistakable symptoms lasting several hours. Spontaneous cures have already been described by Theiler, Green and Viljoen [1915] and quite recently by Kartascheffsky [1926]. However, as such observations are scarce it seems worth while to give here a more detailed description of an interesting phenomenon. It must be emphasised that the temperature of the animal room being more or less constant at 65–68° F. the possibility of "heat cures" [Peters, 1924] is excluded. Nor was there any probability of a contamination of the diet with a vitamin B-containing substance as the symptoms cleared up at

a time when no food was being consumed by the pigeons. Pigeon I *a* looked quite normal on the day following the convulsions, when an X-ray picture of its gastro-intestinal tract was made. For that purpose a barium meal consisting of 5 g. $BaSO_4$, 5 g. of the synthetic diet and 10 cc. of water was administered to the pigeon. It must be pointed out that the barium meal was given after the spontaneous disappearance of the acute symptoms. The pigeon was then quite normal during 5 days and ingested voluntarily during that period 26 g. of the diet. On the sixth day it developed severe acute symptoms—opisthotonos and convulsions associated with impaired respiration—when the bird was killed. The control pigeon II *a* was killed on the following day. Pigeon I *b* developed convulsions and emprosthotonos on the 32nd day of the experiment; it was better on the following day; the symptoms reappeared, however, when the bird was taken out of the cage for weighing. Two days later the pigeon was found in convulsions during the morning inspection of the cages and was killed. The control was killed on the following day. No food had been taken during that period by pigeon I *b*. Pigeon I *d* developed acute symptoms on the 25th day; the pigeon was decidedly better on the following day, but head retraction and a fit of convulsions were provoked by mild disturbances of equilibrium. An X-ray picture was taken the next day and a fit of convulsions was also observed during the process. The following three days were uneventful, but the pigeon died suddenly on the 4th night, no food having been ingested after the first onset of convulsions. The control pigeon died a few hours earlier. Pigeon I *e* developed very severe symptoms on the 25th day of the experiment, "cartwheel" turning and convulsions lasting 7 hours with short intervals. The condition improved in the evening and the pigeon looked much better on the following day. An X-ray picture was taken on the next day. The following 7 days were uneventful. On the 5th day after the first attack the pigeon began to eat (no food having been taken for 6 days up to that time) and ingested 16, 13, 7, 12 and 10 g. daily respectively. On the 10th day after the first attack it developed acute symptoms and was killed. The control bird died on the 30th day of the experiment. The very interesting question of spontaneous cures is being now more fully investigated on a larger number of pigeons.

Temperature.

A progressive fall of the body temperature of pigeons on vitamin B-deficient diets has been observed by many investigators [Dutcher, 1918; McCarrison 1921; Farmer and Redenbaugh, 1925; Barlow, 1926, and others], and those findings are fully confirmed by the present work. Sub-normal steadily decreasing temperatures were also noted by McCarrison in starving birds. The same was found true for the control pigeons of Lot II. The decrease of temperature is roughly equal for both lots up to the onset of acute symptoms. It seems clear, however, that the thermal disturbances occurring during the acute nervous symptoms are of a more complicated character. Spontaneous cures

occurring in four pigeons gave a good opportunity for studying them in detail. Pigeon I *a* had a temperature of 102·2° (Fahr.) on the 23rd day of the experiment and some leg weakness could already be observed. The temperature was 100·4° on the 25th day and 99·5° on the 26th when convulsions occurred. The following day, when the symptoms cleared up, the temperature rose to 104°, *no food having been ingested*. In the morning of the day when the second attack occurred the temperature was 102·9°, it was 91° during the convulsions—a fall of 11·9° (Fahr.) in the course of a few hours. The temperature was unaltered during the first attack of pigeon I *b* (104°) and remained constant to the second. Pigeon I *d* had 104·3° on the 23rd day and 103·1° on the 25th during the convulsions. The temperature rose subsequently and attained 105·1° on the 29th day, on the 30th day it was only 102·5° and the pigeon died in the night (second attack?). Pigeon I *e* had a temperature of 101·4° before the first attack and 99·9° during it, the temperature was 103·2° on the following day, and 104·2° on the 32nd day and then began to drop in spite of a liberal food intake, falling to 101·8° on the 34th day and to 93·7° on the 35th, when the second attack occurred. It is obvious that the control of body temperature is greatly disturbed during the phase with which acute symptoms are associated. A comparison between both lots of pigeons is therefore scarcely justified, but a few average values have been calculated, the temperatures of pigeons having acute symptoms not being included. The data are presented in Table III.

Table III.

Day of Exp.	23	24	25	26	27	28	29	30	31	32	33	34	35	
Lot I	103·5°	—	104·5°	104·2°	104·0°	104·3°	104·0°	103·2°	104·2°	104·0°	104·0°	104·3°	104·2°	Fahr.
Lot II	—	105·2°	—	105·6°	104·7°	—	105·1°	105·0°	105·8°	105·2°	104·9°	105·0°	105·0°	Fahr.

The average temperature is slightly higher in Lot II, but no stress can be laid upon this difference in view of the normal variations that may be encountered. Nevertheless, it seems fair to assume that the thermal changes occurring in pigeons during vitamin B deficiency are twofold; firstly, a progressive decrease which is most probably due to insufficient food intake, and secondly, a profound and rapid fall manifesting itself in the course of a few hours and associated with the onset and gravity of acute nervous symptoms. There seems a possibility that this phenomenon is more directly related to vitamin B. It will be remembered that Farmer and Redenbaugh [1925] observed during convulsions temperatures as low as 92·0° rising to 104° in the course of 6 hours after administration of 0·5 g. of yeast concentrate only. A temperature in the neighbourhood of 103–104° is probably normal for the state of partial starvation occurring in vitamin B-deficient pigeons or in similarly fed controls.

Examination of the alimentary tract by X-rays.

The important work of Cowgill and his colleagues [1921; 1923, 1, 2; 1925, 1926] demonstrates clearly that gastric atony is a characteristic symptom in dogs maintained on vitamin B-deficient diets. "Marked retardation and stasis" is reported from cases of human beriberi by Japanese clinicians [Ohomori *et al.*, 1922]. A study of the deficient pigeon in that respect seemed therefore important, and observations of the rapidity of the passage of a barium meal through the alimentary canal of the deficient birds and the controls were made. This was done on the 27th day of the experiment, almost simultaneously with the onset of acute symptoms in five of the pigeons of Lot I. A barium meal consisting of 5 g. $BaSO_4$, 5 g. of the deficient diet and 10 g. of water was placed in the crop by means of a syringe.

An X-ray picture was then taken 1 hour 35 minutes after the meal; that period of time having been determined as the most suitable by a number of observations on normal birds. The control pigeons were treated similarly on the following day. The results were striking in that no appreciable deviation from the normal could be seen in the deficiently fed pigeons. It is impossible to reproduce here all the photographs, but a few typical ones are presented in Plates III and IV. The picture of pigeon I *d* ((4), Plate IV), deserves special attention, because acute symptoms (convulsions and opisthotonus) were marked whilst the photographs were being taken, so that it was necessary to hold the strongly retracted head in position in order to secure a satisfactory picture. Nevertheless, the food passed through the alimentary canal with normal rapidity, and the strong action of the gut, marked in the photograph, could be most readily followed on the screen. Faeces containing $BaSO_4$ were passed at the same time as by the control pigeons. Many photographs of normal pigeons were also taken but no appreciable differences between them and those of deficient birds or controls could be detected. Unfortunately the control II *d* behaved abnormally, in that no food passed in the gut after 1 hour 35 minutes. This bird died 2 days after the X-ray picture and was most probably ill when the picture was taken.

These results clearly seem to indicate that anorexia in the voluntarily feeding vitamin B-deficient pigeon is not necessarily connected with or accompanied by impaired motile function of the alimentary canal. The question of hunger contractions of the pigeon's stomach is naturally left untouched by the X-ray picture method of investigation. It will be seen later that these results are different from those obtained when the pigeons were fed forcibly on a B-deficient diet.

Histological examination of nervous system.

Pathological changes of the nerves in pigeons fed on vitamin B-deficient diets have been described by very many investigators. It was, however, very soon pointed out by Schnyder [1914] that the rapid disappearance of the characteristic symptoms under the influence of vitamin administration can

Pigeon *a*, Lot I, 1 hr. 35 mins. after $BaSO_4$ meal.

Pigeon *a*, Lot II, 1 hr. 35 mins. after $BaSO_4$ meal.

Pigeon *h*, Lot I, 1 hr. 35 mins. after $BaSO_4$ meal.

Pigeon *h*, Lot II, 1 hr. 35 mins. after $BaSO_4$ meal.

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(1)

Pigeon *a*, Lot III, 1 hr. 35 mins. after $BaSO_4$ meal.

(2)

Pigeon *a*, Lot II, 1 hr. 35 mins. after $BaSO_4$ meal. Given 1 g. yeast extract daily for 6 days after showing acute symptoms.

(3)

Pigeon *a*, Lot IV, 1 hr. 35 mins. after $BaSO_4$ meal.

(4)

Pigeon *d*, Lot I, 1 hr. 35 mins. after $BaSO_4$ meal. X-ray picture taken during convulsions.

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scarcely be due to the repair of degenerated nervous tissue. A similar view is clearly expressed by McCarrison [1921]. Histological examination of pigeons I *f*, II *f*, II *g* and III *d* was kindly undertaken by Dr Woollard in the Department of Anatomy, University College, who reports as follows:

"An histological examination was made of a pigeon that had been on a deficient diet (*f*, Lot I) for 35 days. The bird had exhibited no nervous phenomena during life and an examination of the intermuscular nerves and nerve endings showed an abundance of normal nerves and endings. In fact no deviations from the normal could be recorded in this case. Two control pigeons (*g* and *f*, Lot II) which had received the same amount of food as the birds on the deficient diet voluntarily consumed were also examined. These birds had also been given yeast extract. The examination of the first bird of this group showed perfectly normal intermuscular nerves and nerve endings. The bird was fairly well nourished. The second bird of this series was extremely wasted, and in this case there could be observed considerable changes in the terminal nerves and endings in the muscles. In all cases the tissue examined came from the muscles of the leg. A pigeon from Lot III (*d*) was also examined, it having exhibited typical head retraction for some days. This forced movement can easily be overcome and the head made to assume a normal position. However, the slightest stimulus brings about a reproduction of the retraction. When the bird is held head downwards, the head under the influence of gravity will gradually sink, but again the slightest stimulus will restore the retraction. Such a position involves alteration of the tone in various muscle groups. There is no paralysis and the position of the head bears most resemblance to the forced movements that can be elicited from the labyrinth, from the mid-brain, and from the cortex. Indeed there is little neurological ground for supposing that head retraction depends on changes in the nerves of the neck muscles. The examination of intermuscular nerves in this case was unfortunately not entirely satisfactory, but the preparations were sufficient to show large numbers of normal fibres and endings. To what extent there were degenerative changes it is not possible to say from the specimens. Of the series studied it is to be remarked that the specimen which showed the most advanced changes was the bird that had received vitamin *plus* a quantity of food equal to the voluntary intake of the birds on the deficient diet."

It seems evident from this study that starvation and the wasting associated with it are largely if not entirely responsible for the pathological nerve changes observed in vitamin B deficiency in pigeons. This conclusion provides further support for the views recently put forward by Woollard [1927]. It cannot be sufficiently emphasised that no nerve regeneration is possible in the course of a few hours, and that the nervous symptoms, vanishing so rapidly under the action of vitamin B or even disappearing spontaneously must be due to a quite different cause. There is no logical impediment, however, in assuming that the paralytic form of pigeon's beriberi which is not curable or very hardly curable by vitamin administration may be associated with nerve degeneration.

Blood-sugar.

Funk and Schönborn [1914], Funk [1920], Randoin and Lelesz [1925], Collazo [1923], and others observed hyperglycaemia in pigeons on vitamin B-deficient diets. Funk [1914] considers vitamin B as specially connected with the carbohydrate metabolism. Control determinations on partially starving pigeons were done by Collazo [1923], who found in such pigeons much the same increase of blood-sugar as in pigeons on vitamin B-free diet. He also cites several papers dealing with starvation-hyperglycaemia.

The blood-sugar was estimated in four pigeons of Lot I and in four pigeons of Lot II by the reliable method of Hagedorn and Jensen and the following figures were obtained.

Table IV.

Lot I		Lot II	
Pigeon No.	Blood-sugar	Pigeon No.	Blood-sugar
I *e**	0·214 %	II *b*	0·251 %
I *f*	0·222 %	II *f*	0·224 %
I *g*	0·222 %	II *g*	0·248 %
I *h**	0·228 %	II *h*	0·186 %
Average	0·221 %	Average	0·227 %

* Blood taken during convulsions.

There is no difference between the two lots of pigeons. The blood-sugar content of the normal pigeon is 0·21 % according to Collazo [1923], and 0·185 % according to Honeywell [1922]. The slight increase observed in Lots I and II is most probably due to inanition.

Adrenaline content and weight of adrenals.

A hypertrophy of the adrenal glands in vitamin B-deficient pigeons was first observed by McCarrison [1921] and his observation has been confirmed by many investigators. McCarrison found also an increased output of adrenaline in vitamin B deficiency and considers it as playing a part in the production of oedema in cases of "wet" avian and human beriberi, whilst Funk [1924] ascribes to it a rôle in the causation of hyperglycaemia. It will be remembered that hypertrophy of the adrenals was also repeatedly observed in cases of inanition [Vincent and Hollenberg, 1920; McCarrison, 1921 and Findlay, 1921], but, on the other hand, pure starvation is always associated with vitamin B deficiency. Beznak [1923], while confirming the hypertrophy of the adrenals in beriberi pigeons, could find no increase in the size of adrenals in starving pigeons receiving only vitamin B (in the form of yeast). He therefore considers that hypertrophy of the adrenals is definitely caused by vitamin B deficiency, but points out, since he could find no increase in the adrenaline content, that probably the cortical tissue alone hypertrophies.

The results of a few determinations made on pigeons from Lots I and II (two determinations in each lot) are given in Table IV. The method of Baker and Marrian [1927] was used.

Table V.

	No. of pigeon	Weight at beginning g.	Weight at end g.	Weight of adrenals g.	Adrenaline $\times 10^{-2}$ mg.	Adrenaline in mg./g. gland
Lot I	I *h*	383	233	0·0691	17·8	2·57
	I *e*	457	281	0·0532	11·85	2·23
Lot II	II *h*	355	220	0·0395	17·6	4·45
	II *b*	443	298	0·0352	11·4	3·24

As far as these figures go they tend to confirm the observations of Beznak, but they are obviously inadequate and further work seems desirable before the conclusion is adopted that the hypertrophy of the adrenals is due specifically to a deficiency of vitamin B.

Blood-calcium.

A few estimations of blood-calcium were made with the view in mind that the convulsions might possibly be associated with a hypocalcaemia. Actually a slightly increased amount was found present in the blood of the pigeons lacking vitamin B. The blood-calcium was determined by the Clark-Collip [1925] modification of the excellent Kramer-Tisdall [1921] method. The blood was drawn from the wing vein.

Table VI.

	No. of pigeon	Blood-calcium mg./100 c.c.	Remarks
Lot I	I *a*	12·1	Blood taken during convulsion
	I *e*	11·4	„ „ „
	I *f*	10·0	On 36th day of vitamin B-free diet
Average: 10·9	I *g*	10·0	„ „ „ „
Normal aver. 8·2	Normal	8·2	
	„	8·2	
Lot II	II *a*	8·3	
	II *b*	8·4	
Average: 8·3	II *g*	8·3	

Ungar [1927] reports no appreciable difference between the blood-calcium levels in normal pigeons and others during beriberi convulsions.

Starch-hydrolysing enzymes of the alimentary tract.

A complete loss of the amylolytic and lipolytic, and a marked decrease of the proteolytic enzymes in pigeons fed on vitamin B-deficient diets has been reported by Farmer and Redenbaugh [1925]. Previously various investigators tried to connect the outstanding features of vitamin B deficiency with changes in the quantity and activity of the digestive juices. That no direct relation exists between vitamin B and the secretory function of the digestive glands has been conclusively demonstrated by the work of Anrep and Drummond [1921] and Cowgill and colleagues [1921 to 1926]. On the other hand, it appeared to us that the almost identical behaviour of the pigeons in our Lots I and II was hardly compatible with the findings of Farmer and Redenbaugh. It seemed, therefore, important to repeat their work. This was done, using three normal pigeons kept previously in a large aviary on poultry mixture diet, four pigeons of the deficient Lot I, one pigeon of the deficient Lot III and four pigeons of the control Lot II. The technique of Farmer and

Redenbaugh was closely followed except that the pancreas and first 6 inches of the intestines were not minced in a meat mincer but ground with sand in a mortar. The glycerol extracts were centrifuged after 4 weeks' maceration and the amylolytic properties determined according to Long and Johnson [1913]. The results are given in Table VII.

Table VII. *Starch digestion.*

Incubation at 37°.

Pigeon No.	After 10 mins.	After 45 mins.	After 80 mins.	After 120 mins.
Normal pigeons.				
Normal I	blue	blue	purple-red	red
,, II	blue	blue	purple	purple-red
,, III	blue	purple	pale pink	very pale pink
Deficient pigeons (Lot I and Lot III). All killed during convulsions with the exception of III *h*.				
I *a*	blue	purple	pale pink	very pale pink
I *b*	blue	faint yellow	faint yellow	faint yellow
I *e*	purple-blue	faint yellow	faint yellow	faint yellow
I *h*	blue	purple-red	faint yellow	faint yellow
III *h*	blue	purple-red	faint yellow	faint yellow
Controls (Lot II).				
II *a*	blue	blue	reddish purple	red
II *b*	blue	purple	pale purple-red	faint yellow
II *d*	blue	faint yellow	faint yellow	faint yellow
II *h*	blue	purple-blue	pink	very pale pink

Our observations are entirely different from those made by Farmer and Redenbaugh, but are in agreement with other findings reported in the present paper, all of which demonstrate clearly that the digestion, at any rate of starch, is normal in a vitamin B-deficient pigeon. The extracts of the organs of the normal pigeons are, if anything, rather less active than those of the deficient birds or of the controls. This somewhat unexpected result is probably explained when one takes into consideration the great wasting which the deficient birds have suffered, so that the extracts are simply more concentrated, as the wasting involves chiefly inactive material such as the fat of the pancreas and muscular wall of the gut. It must be remembered that in the experiment of Farmer and Redenbaugh the birds were fed forcibly and that they were eating voluntarily in the present experiment. A highly active extract was, however, obtained also in the case of pigeon III *h* belonging to a group that had been forcibly fed. It is not easy to account for the results of Farmer and Redenbaugh, but what is believed to be an inconsistency in their results may here be pointed out. It seems very difficult to understand how pigeons having, according to their opinion, completely lost the power of utilising starch and fats and to a marked extent that of splitting proteins could be kept for an average period of 27 days on a vitamin B-free diet of autoclaved grain with an average loss of only 3·7 g. daily. The average daily food intake was 25 g. Even leaving out of consideration the alleged partial impairment of the utilisation of proteins and total of fats, and only deducting from that figure 70 % for non-metabolised

starch it is easy to calculate that only 7·5 g. of the diet containing a considerable amount of roughage were in reality available for the pigeons. That quantity was furthermore greatly diminished by the fact that pigeons "frequently regurgitated a considerable amount" and was surely not sufficient to spare daily 5·6 g. of body tissue. A similar recalculation in the case of the lard diet fed in quantities of 15 g. daily and containing 39 % of starch and 21 % of lard would lead, if the assumptions of Farmer and Redenbaugh were correct, to an even more striking result as the daily loss in that group of pigeons was only 1·17 g. (McCarrison's figures for starvation are 9·3 g.). It will be remembered that the control pigeons of Lot II receiving a liberal supply of vitamin B in the form of marmite and with an average daily food consumption of 6·1 g., none of which was regurgitated, presented an average daily loss of 5 g. of weight. There is also another statement in the paper of Farmer and Redenbaugh with which we cannot agree, namely, that "the gastro-intestinal tract of the starved pigeon is normal and empty with little or no permanently decreased efficiency of the digestive enzymes. In the pigeons with polyneuritis ...the gut usually shows degenerative changes." That the gastro-intestinal tract undergoes very marked changes in starvation and can by no means be considered as normal has been established beyond doubt by the researches of McCarrison on a great number of pigeons.

Lots III and IV.

Pigeons fed forcibly with a vitamin B-deficient diet almost invariably vomit a great deal of it, so that accurate estimates of food intake are difficult to make. Similar rejection of food is sometimes seen in birds feeding voluntarily on the deficient diet, but was not observed in our Lot I. Such experiments are therefore much more liable to error than those carried out when the ingestion is voluntary. It is also, in our opinion, desirable that the abnormal method of feeding, particularly when applied to an animal that exhibits a distaste for the particular food, and its possible effects on the animal should be always borne in mind when the results of such experiments are being discussed. This applies to the results obtained in the present work with Lots III and IV so that they will be only briefly treated. Two interesting features must, however, receive attention. The two lots of pigeons were fed daily 20 g. of the finely ground diet mixed with a sufficient amount of water to permit passage through the syringe and rubber tube. Approximately 30 cc. of water were used. Pigeons of Lot IV were also given daily 1 g. of marmite. The pigeons of the deficient group began to regurgitate the food on the 5th to 9th day of the experiment. The vomiting was purely voluntary and was effected in the same manner as when feeding the young. Some birds vomited immediately after being fed, some only after a few hours had elapsed. Vomiting occurred also to a certain extent in the control Lot IV and was most probably due here to the discomfort caused by the large bulk of food being introduced at once in the crop. It must be emphasised here that vomiting never occurred in the

control Lot II—the quantities of food administered being much smaller than in the case of Lot IV. A marked retention of the food in the crop was always observed in the deficient pigeons in the later stages of the experiment and especially just prior to the onset of the acute symptoms when they were unable to execute the movements of the head and wings necessary to expel the contents of the crop.

Weight.

The initial average weights and the average weights on the 18th day of the experiment of both lots are given in Table VIII. After that date food retention associated with acute symptoms was occurring frequently and the weight figures were therefore unreliable.

Table VIII.

	Initial weight	Weight on the 18th day	Average daily loss
	g.	g.	g.
Lot III*	417	330	5·1
Lot IV	416	388	1·6

* Average of six pigeons—III *e* died on the 13th day of the experiment and III *c* developed acute symptoms and was given marmite on the 15th day.

The loss in weight of the pigeons of Lot IV was due to the occurrence of vomiting and not to an insufficient food intake, as gain in weight was observed in those pigeons which did not vomit.

It is very interesting to note that the average daily loss of Lot III is only slightly lower than that of Lot I—forcibly fed pigeons practically behave as if they were feeding voluntarily in the respect that only a certain definite amount of the food deposited in the crop is allowed to pass further down the alimentary canal and the remainder is expelled.

X-ray picture.

X-ray pictures of the gastro-intestinal tract of pigeons III *a*, III *b*, III *c* and their controls, and III *f*, III *g*, III *h* and their controls, were made under conditions quite similar to those already described on the 12th and 15th days of the experiment respectively. A marked difference between the deficient lot and the controls could be observed in all the cases. It is illustrated in two typical photographs of pigeons III *a* and IV *a* reproduced here ((1) and (3), Plate IV). 1 hour and 35 minutes after the meal the stomach alone is faintly visible in the deficient pigeon and no food is seen descending the oesophagus: in the control pigeon the oesophagus can be easily detected, the stomach and intestines are full and the barium meal is approaching the rectum. Pigeon III *a* was then given daily marmite, beginning with the 18th day of the experiment, and an X-ray photograph was taken on the 24th day showing normal passage of the food through the alimentary tract ((2), Plate IV). The retardation of the passage of food in the pigeons that have been forcibly fed might be due to an impairment of the mechanism for emptying the crop in a normal manner or

to a voluntary inhibition of this process. It might also be due to an absence of the stomach movements, and to decreased peristalsis of the intestines. We are inclined to favour the first view, for the reason that any food passing to the intestines was normally digested, and normal peristaltic movements could be seen on the screen when after a sufficient period of time the Ba meal was present in the intestines. If the crop was functioning normally a passive filling of the empty intestine a short time after administration of the Ba meal could be expected. It is believed that the impaired functioning of the crop is not due to muscle atrophy or nerve degeneration, because the condition improves rapidly as was observed by many investigators after administration of vitamin B in a suitable form.

Acute symptoms.

The acute symptoms occurred sooner in Lot III than in Lot I. Four pigeons, III *e*, III *d*, III *f*, III *g*, presented the typical picture after 15, 37, 19 and 22 days respectively with an average of 23 days as compared with 28 days in the case of those voluntarily feeding. Two other pigeons, III *a* and III *b*, developed paralysis and leg weakness, but no symptoms of head retraction after 17 days and 22 days. III *a* quickly recovered after administration of marmite, but III *b* died on the following day. Pigeon III *c* died from other reasons on the 13th day of the experiment. Pigeon III *h* was killed on the 26th day without showing acute symptoms.

Discussion.

Reviewing the results of this investigation as a whole it seems apparent to us that many of the characteristic symptoms exhibited by pigeons fed on diets deficient in vitamin B are direct consequences of the failure to consume sufficient food to maintain the body weight. In this respect these experimental results are confirmatory of those reported by Drummond and Marrian [1926] derived from somewhat similar studies of the rat. In that paper evidence was presented to disprove a number of theories ascribing to vitamin B an essential influence on the production or functioning of the oxidative mechanisms of the tissues. It is believed that the present paper serves to throw doubt on another group of theories, namely, those which relate vitamin B to the digestive and assimilatory functions of the organism. In the first place, the view expressed by various authors that vitamin B is necessary for the proper utilisation of carbohydrates is unsatisfactory in that it ignores the fact that more than 50 % by weight of the molecule of proteins is metabolised along the path of carbohydrate breakdown. Such a view implies that the mechanism responsible for the utilisation of carbohydrates arising from proteins remains normal in vitamin B deficiency, whereas a distinct and independent mechanism involved in the metabolism of exogenous carbohydrates undergoes a profound disturbance. It is very difficult on that basis to account satisfactorily for the results of Randoin and Simonnet [1924, 1, 2], who reported that they were able to keep pigeons in perfect health for a period of $3\frac{1}{2}$ months on a diet deficient in vitamin

B and in carbohydrates but containing 54 % of protein and 32 % of fat, or on a diet almost identical to that used in the present work[1], but fed in amounts of 75 g. per pigeon daily, from which the pigeons were able to "pick out" the protein and other constituents, leaving the carbohydrate part untouched. Why a reduction of the daily intake of that diet to 20 g. should hasten the onset of acute symptoms—which then appeared after 2 months—remains obscure. A similar difficulty is encountered when discussing the work of Funk and his colleagues [Funk, 1914; Funk and Paton, 1922; Collazo and Funk, 1924] on the subject. In a recent paper Funk and Collazo [1925] report that pigeons receiving a constant amount of vitamin B and kept in weight equilibrium on a diet containing 12·5 g. protein showed an increase in body weight roughly proportional to the amount of protein ingested when offered dietaries containing 25, 50 and 75 % of protein, the other variable component being starch. It seems clear that in these cases the food values of the diets and their influence on the weight curve were not properly controlled. A review of our experimental observations makes it evident that no results corroborating these theories have been obtained. The actual food intake of a pigeon from which vitamin B is withheld, be it forcibly fed or partaking freely of the diet, is greatly diminished, but the ingested quantum is metabolised just as completely as in a pigeon receiving liberal amounts of vitamin B in the form of yeast extract; and this is true even in the stage of the deficiency disease where the characteristic convulsions appear. The similarity of the curves for food intake and body weight, the similar rates of passage of a meal through the intestines, the blood-sugar levels and composition of the faeces all support this view. Furthermore, it is believed that the diminished food intake and subsequent partial starvation are sufficient to explain any hyperglycaemia encountered in vitamin B-deficient pigeons. This confirms the findings of Collazo [1923], but the views expressed by Funk and Schönborn [1914], Funk [1920], Randoin and Lelesz [1925] and others cannot be shared. In fact, the starvation factor was not taken into account by these investigators. Passing now to the relation of vitamin B deficiency to the function of the digestive glands it is obvious that a normal digestion of starch observed in beriberi pigeons suggests the existence of a normally functioning digestive apparatus. This view is supported by the demonstration of the presence of a highly active amylolytic enzyme in the pancreas and intestines of beriberi pigeons, confirming the finding of Tiger and Simonnet [1921] and Rothlin [1922]. That the gastric secretion has a normal composition throughout the course of vitamin B avitaminosis has been conclusively demonstrated by Bickel [1922]. Anrep and Drummond [1921] and Cowgill and Mendel [1921] presented experimental data leading them to deny any stimulating influence of vitamin B on the digestive glands. It seems that the evidence weighs against the opinion and findings of Farmer and Redenbaugh [1925].

[1] Randoin and Simonnet used potato starch instead of rice starch and reach the curious conclusion that a vitamin B-deficient pigeon can digest the latter but not the former.

We have been greatly disturbed during this work by the number of spontaneous cures of the typical beriberi convulsions that have occurred. In our opinion such cures are much more frequently encountered in pigeons fed on an artificial diet such as we have employed than in those fed in the more customary manner on polished rice.

That a substance indistinguishable in all its characteristics from what we are accustomed to call vitamin B is present in the organism under normal conditions seems certain. Even after a long period of vitamin B deficiency the body stores are not completely exhausted as was shown by the interesting work of Funk [1914], since confirmed by various authorities [Theiler, Green and Viljoen, 1915; Hess and Takahashi, 1921; Abderhalden, 1922]. It will be remembered that Funk extracted the tissues of pigeons after death in beriberi convulsions in a manner similar to that used for extracting rice polishings and obtained extracts which had marked curative effects when administered to other pigeons in convulsions. That result recalls the observation of Baker, Dickens and Dodds [1924], that large quantities of insulin can be extracted from the organs of patients dying in diabetic coma.

There is a possibility that there may be a liberation of the reserves of vitamin B in the tissues during periods when there is none in the diet and when there is considerable tissue wasting; that such liberation may occur is suggested by the fact that pigeons and fowls do not exhibit in pure starvation (which is also obviously a vitamin B deficiency) the characteristic nervous symptoms of vitamin B deficiency unless they are "washed out" by administration of large quantities of water [Eijkman and Hoogenhuyze, 1916; Chamberlain, Bloombergh and Kilbourne, 1911; Chamberlain and Vedder, 1911]. The question immediately arises whether what is perhaps the most characteristic symptom of vitamin B deficiency, namely, the loss of the desire to partake of the deficient food, is to be looked upon merely as a morbid phenomenon or as an attempt of the organism to liberate by starvation and subsequent tissue wasting the body stores of the lacking vitamin bound in the tissues so as to be inaccessible to the animal under normal conditions and thus make available a substance essential for life. The occurrence of spontaneous cures on diets such as we employed suggests that there may be a liberation following a convulsive seizure which gives temporary relief and sometimes leads to a transient return of appetite. Further investigation must follow before an opinion on this question can be formed. It seems evident that almost all the pathological changes observed in beriberi pigeons are directly attributable to inanition. There are, however, certain differences between a vitamin B-deficient pigeon and one partially starving but receiving a liberal supply of the vitamin, namely, the occurrence in the former of two well-defined symptoms which in the order of their appearance are: (1) a diminished desire to partake of *the deficient food*, (2) symptoms known as opisthotonos, emprosthotonos, convulsive seizures, "cartwheel" turning. Leg weakness is also frequently present, but is not characteristic as a symptom. It yields only with difficulty to vitamin B treatment and is often observed in the premortal stage in control birds.

Regarding the fluctuations of temperature that are associated with the convulsion phase of vitamin B deficiency which seem of different origin from the fall that is a consequence of inanition, and the hypertrophy of the adrenals, we are not yet prepared to express an opinion. Much evidence is against associating the nervous symptoms described under (2) with the nerve lesions considered by many authorities as a characteristic consequence of vitamin B deficiency. Identical lesions have been found by Dr Woollard on histological examination of our control pigeons on restricted food but receiving a liberal supply of yeast extract, whereas they are often lacking in beriberi pigeons (McCarrison). Finally, the prompt recovery following administration of vitamin B excludes any possibility of nerve regeneration and strongly suggests that the mechanism responsible for the appearance of the symptoms or for their retrogression is of a different character.

It may be stated with some certainty that no existing theory is adequate satisfactorily to explain the origin of the nervous syndrome characteristic of vitamin B deficiency or the rôle played by vitamin B in the normal organism. As yet we wish to make no contribution to the theoretical side; we would, however, support the opinion of Karr [1920] that "some relationship exists between the desire to partake of food and the amount of the so-called water-soluble vitamin ingested."

Summary.

(1) A method of studying vitamin B deficiency is described in which the effects of inanition are checked by a suitable control. The method, as applied to pigeons, consists in determining their voluntary food intake when kept on a vitamin B-deficient but otherwise complete synthetic diet and administering artificially the same amount of food to individual control birds of the same weight which receive in addition a liberal supply of vitamin B in concentrated form.

(2) As a result of such studies the decrease in body weight accompanying vitamin B deficiency in pigeons is considered as solely attributable to partial starvation.

(3) No difference could be observed in the utilisation of food between the deficient birds and the controls.

(4) Green faeces are considered to be due to insufficient food intake and possibly bacterial infection.

(5) The "ruffled feathers" attitude of the deficient birds is due to inanition.

(6) No difference was found between the blood-sugar levels of the two groups. The hyperglycaemia, if any, is due to inanition.

(7) A hypertrophy of the adrenals in vitamin B deficiency is considered as probable, but no difference in the adrenaline content is found.

(8) No difference could be observed in the rate of passage of a barium meal through the gastro-intestinal tract of birds feeding on the deficient diet voluntarily and of the controls receiving the vitamin.

(9) A marked slowing of the rate of passage of the barium observed in the case of artificially fed birds is attributed not to intestinal stasis but to a "barrier action" of the crop.

(10) The gradual fall of the body temperature observed in deficient pigeons is due to partial starvation.

(11) A loss of temperature control during the convulsive stage of beriberi is considered as probable.

(12) Highly active amylopsin preparations are obtained from the pancreas and intestines of deficient birds killed during convulsive seizures, as well as from controls.

(13) The view that vitamin B controls the carbohydrate metabolism is not corroborated.

(14) Lack of appetite for *the deficient food* and acute nervous symptoms are characteristic of vitamin B deficiency in the pigeon.

(15) No relation could be traced between the occurrence of acute nervous symptoms and degenerative changes in the nerves. Such changes were present in both groups of pigeons and are attributable to inanition.

(16) No existing theory is considered as giving a satisfactory explanation of the rôle of vitamin B in the normal organism, or of the true nature of the conditions induced by its deficiency.

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LXXXIX. THE PHYSIOLOGICAL RÔLE OF VITAMIN B. PART IV.

THE RELATION OF CERTAIN DIETARY FACTORS IN YEAST TO GROWTH OF RATS ON DIETS RICH IN PROTEINS.

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Introduction.

Drummond, Crowden and Hill [1922] reported that rats and cats showed a subnormal rate of growth when fed on diets composed largely of caseinogen, but containing what were considered to be adequate amounts of the inorganic salts and vitamins. These observations seemed definitely related to those of Hartwell [1921, 1924, 1925] made during an investigation of mammary secretion in rats, in which she traced a quantitative relation between the protein level of the diet and some factor or factors showing a similar distribution in foodstuffs to vitamin B. A somewhat similar conclusion was reached by Reader and Drummond [1926] who obtained satisfactory growth in rats only when the ratio of protein to the yeast extract used in the diet was about 5 or less.

A consideration of these and other results did not lend support to the opinions that have been expressed to the effect that the vitamin requirement stands in relation to the amount of carbohydrate ingested or metabolised, and provisional support was given to Hartwell's view that it is concerned with the fate of the proteins or their constituent units as a whole rather than with the metabolism of the nitrogen-free residue as was at one time suggested. Another suggestion has been advanced by McCollum and Simmonds [1925] who ventured the view that the subnormal rate of growth noted in our experiments was due to an improper balance between the calcium and phosphorus in the diet, leading to "high-phosphorus" rickets. Such a possibility had suggested itself to us, especially as in the first set of experiments some lameness was noted in the experimental cats, but Reader and Drummond were unable to detect any failure of calcification on X-ray examination of the rats reported in the paper published in 1926. Furthermore, it seemed

unlikely that supplementing the protein-rich diet with additional yeast extract itself rich in phosphorus and poor in calcium could improve the balance of these elements.

It is, therefore, not by any means clear what the action of the yeast extract is in these experiments and further tests were made in order to throw some light on the matter.

Some of Hartwell's results point to the yeast factors operating in her experiments being organic in nature. She was unable to reproduce the effect of the yeast extract by adding salts; the failure of calcium being significant in the light of McCollum's suggestions. Nevertheless, it seemed desirable to ascertain whether the phenomenon observed by us had the same origin as that studied by her, and to determine whether the yeast extract served to adjust the salt or acid-base balance of the diet; it must be remembered that the metabolism of large quantities of caseinogen calls for the neutralisation of considerable quantities of sulphuric and phosphoric acids.

Another point to be studied was obviously the nitrogen metabolism of animals failing to grow satisfactorily on a diet inadequately balanced as regards protein and the yeast factor, in order to find out whether there was any failure to digest and absorb proteins or a disturbance of metabolism reflected in changes in the distribution of nitrogen in the urine.

It will be convenient to deal with this part of the investigation first.

Experimental.

Nitrogen balance. Three young male rats of the same litter were placed in metabolism cages of the Hopkins pattern. Analyses of the excreta were made every few days, decomposition of the urine having been prevented by collection in dilute sulphuric acid and a small quantity of toluene. The

Table I.

	No. Days in period	Total food consumption Dry wt. g.	Average daily consumption Dry wt. g.	Nitrogen-intake g.	Nitrogen-output		Nitrogen balance g.	Change in wt. of rat g.
					Faeces g.	Urine g.		
Control rat on	4	45·85	11·46	1·9890	0·1364	1·2110	+0·6416	+ 5
Diet *A* (low	4	53·00	13·25	1·9867	0·1073	1·3020	+0·5774	+ 8
protein, low	4	59·99	14·99	1·9378	0·1635	1·3720	+0·4023	+ 7
yeast)	4	68·03	17·01	2·0651	0·1521	1·3685	+0·5445	+10
	5	76·24	15·24	2·4551	0·1786	1·6660	+0·6104	+ 9
Rat on Diet *E*	4	32·18	8·04	3·3051	0·1258	2·7895	+0·3898	+10
(high protein,	4	34·94	8·73	3·5891	0·1416	3·840	−0·4265	−12
low yeast)	4	41·97	10·49	3·6682	0·1330	3·2340	+0·3012	+ 7
	4	44·59	11·14	3·9796	0·1455	3·4577	+0·3764	+13
	4	43·26	10·81	3·8808	0·1489	3·6855	+0·0464	+ 1
Rat on Diet *C*	4	36·01	9·00	3·9287	0·1335	3·052	+0·7432	+15
(high protein,	4	39·31	9·82	4·0695	0·1910	3·780	+0·0785	+ 5
high yeast)	5	46·25	9·25	4·4527	0·2119	4·0135	+0·2273	+ 4
	4	46·36	11·59	4·4569	0·1741	4·284	−0·0012	+ 3
	4	47·05	11·76	4·5234	0·1650	3·983	+0·3754	+ 5

faeces were removed daily, dried at 100°, powdered, and a sample weighed out for analysis.

Three diets of the composition used by Reader and Drummond were employed.

	Low protein, low yeast (*A*)	High protein, high yeast (*C*)	High protein, low yeast (*E*)
Caseinogen	20	70	70
Rice starch	70	20	20
McCollum's salt mixture	5	5	5
Yeast extract	4	16	4
Cod-liver oil	2	2	2

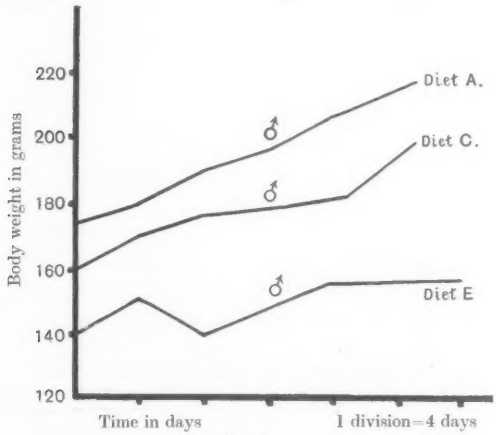

Fig. 1.

The growth of the three animals is shown in Fig. 1, the curves of which are similar in general features to those recorded previously from this laboratory. The analyses and figures in Table I indicate clearly that there is no appreciable disturbance of the digestion and absorption of protein in the case of rats showing unsatisfactory growth on diets rich in protein but with an inadequate amount of the related yeast factor (Diet *E*). The average faecal excretion of nitrogen daily in each group is—Diet *A*, 0·0351; Diet *C*, 0·0417; Diet *E*, 0·0297 g.; figures well within the normal range for animals of this weight.

Distribution of nitrogen in the urine. In this experiment groups of several animals were placed in metabolism cages in order to obtain sufficient urine for a full examination. Four groups were employed, three of which were fed on the diets already described whilst a fourth was given a modified diet containing a normal proportion of protein (20 parts) but the larger amount (16 parts) of yeast extract. This was necessary in order to control the effect of the extra yeast with its high content of nitrogenous substances, purines, etc., on the composition of the urine.

The rats were all males of about 150 g. at the beginning of the experiment and the urines were usually collected and analysed every second day. In some

later experiments only two rats were kept in each cage and the urines were collected over a period of four days. The urinary analyses were made by the following methods. Total nitrogen by Kjeldahl, urea by van Slyke's urease method, ammonia by Folin's aeration process, creatinine and uric acid by the Folin colorimetric methods, amino-acids by the formalin method, allantoin by Handovsky's [1914] modification of Wiechowski's technique; it being found more satisfactory to determine nitrogen in the mercury precipitate than to estimate the excess of that element in the final filtrate by volumetric means. Duplicate analyses were made in practically all cases.

Table II. *Diet A.* (*Control* 1.)

No. of rats	Period Hours	Volume of urine cc.	Total nitrogen g.	Urea-nitrogen g.	Urea-nitrogen % of total N	Ammonia-N g.	Ammonia-N % of total N	Creatinine-N g.	Creatinine-N % of total N	Uric acid-N g.	Uric acid-N % of total N	Allantoin-N g.	Allantoin-N % of total N	Undetermined N g.	Undetermined N % of total N
4	48	80	2·061	1·801	87·39	0·093	4·5	0·0149	0·7	0·0054	0·28	—	—	0·144	7·13
8	—	267	6·845	6·110	89·69	0·197	4·1	0·0416	0·6	0·0145	0·20	—	—	0·482	5·41
4	96	218	4·922	4·576	92·96	0·105	2·14	0·0446	0·9	0·0125	0·25	0·143	2·9	0·0409	0·85
4	72	185	3·942	3·538	89·74	0·0897	2·21	0·0312	0·8	0·0107	0·27	0·164	4·2	0·1084	2·78
4	96	245	5·995	5·258	87·70	0·1612	2·60	0·0405	0·7	0·0121	0·20	0·100	1·7	0·4232	7·1
Av. daily output per 100 g. rat weight (for last 3 experiments only)		8·54	0·1963	0·1767		0·0047		0·00145		0·00046		0·0054		0·0131	

Table III. *Diet E.*

9	48	365	14·460	13·540	93·63	0·4772	3·3	0·0382	0·26	0·0150	0·1	—	—	0·3896	2·71
4	96	410	14·920	14·17	94·95	0·364	2·4	0·0443	0·30	0·0130	0·09	0·138	0·90	0·1907	1·36
4	72	298	10·690	10·01	93·69	0·3142	2·9	0·0274	0·26	0·01	0·09	0·113	1·06	0·2154	2·00
4	96	355	14·01	12·86	91·81	0·4055	2·9	0·035	0·25	0·0125	0·1	0·108	1·15	0·6862	3·79
Av. daily output per 100 g. rat weight (for last 3 experiments only)		15·38	0·5735	0·536		0·0157		0·00155		0·0005		0·0052		0·0199	

Table IV. *Diet A "modified."* (*Control* 2.)

4	120	440	9·363	7·952	84·94	0·282	4·10	0·0493	0·5	0·0201	0·2	0·400	4·3	0·660	5·96
2	96	144	3·377	2·963	87·76	0·131	3·9	0·0226	0·7	0·007	0·2	0·1617	4·8	0·0918	2·64
Av. daily output per 100 g. rat weight		9·9	0·221	0·1913		0·0076		0·0013		0·0005		0·010		0·0093	

Table V. *Diet C.*

4	120	475	15·310	13·73	89·69	0·4543	3·7	0·0297	0·2	0·0139	0·09	0·3144	2·1	0·7677	4·22
2	96	174	7·318	6·674	91·20	0·2606	3·6	0·0195	0·27	0·0067	0·09	0·130	1·8	0·2272	3·04
Av. daily output per 100 g. rat weight		15·65	0·5653	0·511		0·0183		0·0013		0·0005		0·0109		0·0233	

The results are set out in Tables II to V. In the first place it is of interest to note the diuresis that is marked as a result of the high protein diets. The volume of urine passed daily by the rats on these diets was nearly double

that voided by the controls on the low-protein ration. It seems probable that the diuresis is due to the greatly increased urea formation in the former animals.

Apart from this an inspection of the tables reveals very little. On the diets rich in caseinogen there is the expected rise of urea and ammonia nitrogen; in all cases there is the constancy of creatinine and uric acid excretion that one would anticipate, whilst the allantoin excretion reflects the fate of the purine bases that are added to the diet in the form of the yeast extract.

In other words, these analyses reveal no disturbance of the nitrogenous metabolism that could be regarded as a possible cause of the failing nutrition of the rats fed on diets rich in protein and imperfectly balanced as regards yeast.

Attempts to determine the nature of substances responsible for the effect of yeast.

In the first place it seemed essential to ascertain whether the action of the yeast extract was attributable to inorganic or organic constituents. A litter of eight young rats was divided into four groups. Two were fed on the high-protein, high-yeast diet (*C*), whilst two others were fed on the high-protein, low-yeast diet (*E*) to which had been added the ash corresponding to the additional 12 parts of yeast extract that were present in diet *C*. The growth curves of these animals are shown in Charts 1 and 2, Fig. 2, and indicate that the salts present in the ash of the other 12 parts of yeast extract did not serve to permit good growth on the high-protein ration. The question then arose as to how it could be determined whether vitamin B was responsible for the effect we were studying. It is generally accepted that this substance is unstable to alkalis and that the antineuritic and growth-promoting properties of foodstuffs such as yeast are not shown by the usual tests after they have been treated with alkalis. Accordingly a diet was constituted in which 70 parts of caseinogen and 4 parts of yeast extract were supplemented with 12 parts of extract that had been autoclaved at 120° for 1 hour at a strongly alkaline reaction and subsequently adjusted to the original hydrogen ion concentration of the extract by the addition of acid. To our surprise this material was, if anything, even more effective in enabling growth on high protein to be satisfactory than the untreated extract (Chart 3, Fig. 2).

The control tests in which all the yeast extract was given in the form of the material that had been treated with alkali (Chart 4, Fig. 2) demonstrated that no growth was made. The nutritive failure of these two animals was checked and growth resumed when the diet was further supplemented with 5 mg. daily of a concentrate prepared from yeast according to Seidell's method [1926].

These experimental results seemed clearly to indicate that at least two

substances, both organic in character, are responsible for the effect of the yeast and that one is probably identical with the substance that is generally known as vitamin B.

The preparation of the yeast concentrate that showed this effect was placed at our disposal by the kindness of Dr A. Seidell. He has described its preparation [1926] and its power of preventing loss of weight in pigeons in

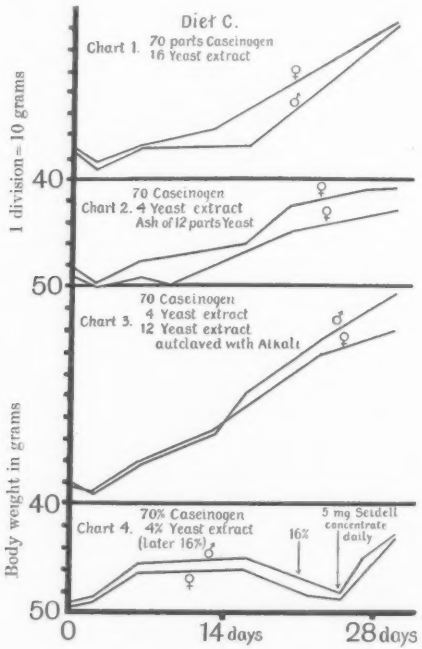

Fig. 2.

doses of about 10 mg. daily. Tested on rats in the usual manner for testing for the presence of vitamin B in foodstuffs we found that the following increments were registered on administering the doses stated:

Dose of yeast concentrate	Change of weight per week
2 mg.	+ 3·5
5 ,,	+ 5·3
10 ,,	+ 7·0
20 ,,	+ 9·7

On the basis that a diet of normal protein content when supplemented with 20 mg. of this concentrate is adequate for steady growth the attempt was made to determine whether increasing the dose of concentrate in proportion to the increase of yeast that had been found necessary would enable good growth to take place on diets rich in protein.

It was found that such was not the case. Doses as high as 80 mg. daily of this preparation did not supplement a diet containing 70 parts of caseinogen as did the equivalent 16 parts of yeast extract.

Convinced from these results that at least two dietary factors derived from the yeast were concerned, we conducted a series of experiments on rats fed on the usual vitamin B-deficient diet employed in the laboratory in order further to trace the relationship between those stable to alkali, and those destroyed by that agent.

The following changes in weight were recorded for a series of pairs of rats of nearly the same initial weight fed on the standard vitamin B-deficient diet with various supplements.

Average weekly alterations in weight of rats fed on vitamin B-deficient ration plus supplements.

Supplement		
Alkali-treated yeast extract % in diet	Seidell's concentrate mg. per day	Change in g.
0	0	− 9·0
0	2	+ 3·5
0	5	+ 5·5
2	0	− 7·5
4	0	− 7·5
2	2	+ 5·7
2	5	+ 5·0
4	2	+10·5
4	5	+26·0

The average weekly increment of rats of this age on the basal diet supplemented with 4 % of untreated yeast extract is about 20 g., so that the group receiving 4 % of alkali-treated extract plus 5 mg. of Seidell's concentrate can be regarded as showing a normal rate of growth.

Discussion[1].

It seems clear from these results that definite indication has been obtained of the existence of two factors in the yeast extract we employed (marmite) that play an important part in regulating growth in young rats. These factors may be differentiated by the action of hot alkali which destroys one leaving the other still physiologically active. It is seemingly the latter that is primarily concerned in the relation between yeast extract and the protein content of the diet that has been traced.

It is possible that our observations provide an explanation of the good growth on diets rich in protein shown by Osborne and Mendel's rats [1921] which received a ration containing two sources of vitamin B, namely yeast and alfalfa.

[1] When this paper was in the press our attention was called by Dr Seidell to the publications of Goldberger and his colleagues [1926] with which we were unfortunately not familiar. In these evidence is presented of the existence of more than one dietary factor of the vitamin type in yeast. The relation between Goldberger's P-P factor, "bios" and our alkali-stable unit awaits investigation.

They are also obviously closely related to those reported by Seidell [1926] who found that autoclaved yeast, in which, presumably, the antineuritic factor had been largely destroyed, supplemented the growth-promoting effect of his preparation of yeast concentrate in a curious manner and also to those published by Smith and Hendrick [1926].

The question of the identity of the so-called antineuritic vitamin with the water-soluble growth-promoting factor has been many times discussed without anything approaching a definite answer having been obtained.

Perhaps the most convincing work recorded in the literature is that of Funk and Dubin [1922] and Funk and Paton [1922] who drew attention to the fact that the rat requires more than one accessory substance of the vitamin B type for growth and well-being. This conclusion was reached as a result of experiments on the fractional adsorption of substances in yeast extracts on the surface of fuller's earth and norit. They believed these substances to be firstly that usually described as the antineuritic factor, and secondly a substance or group of substances responsible for the so-called "bios" effect observed in the growth of yeasts and other micro-organisms[1].

It is not proposed in this paper to review the extensive literature on the question of the number and characteristics of the water-soluble vitamins as in our opinion this can only be done adequately when the results of further investigations are to hand.

Summary.

1. Evidence is presented that the failure to grow at a normal rate shown by rats fed on diets consisting largely of protein (caseinogen) but containing the usual proportion of yeast serving as a source of vitamin B is not due to an impairment of the digestion or absorption of the ingested protein.

2. No signs of an abnormal course of protein metabolism could be detected in such animals by a study of the distribution of the chief nitrogenous constituents in the urine. The increased urea production on the protein-rich diets causes a marked diuresis.

3. The subnormal rate of growth alluded to is apparently due neither to deficiencies in the inorganic constitution of the ration nor to disturbances of the acid-base exchange.

4. The restoration of a normal rate of growth that follows the addition of extra yeast extract is also seen when the extract has previously been treated with hot alkalis so as to inactivate the factor generally known as vitamin B.

5. For a normal rate of growth to be shown by rats fed on diets consisting mainly of protein two factors, which can be differentiated by their behaviour on treatment with alkalis, seem to be necessary.

[1] Funk gave this factor the designation "vitamin D." Unfortunately at about the same time McCollum suggested the title of vitamin D for the antirachitic vitamin. Some slight confusion followed but has now disappeared as a result of the almost general adoption of McCollum's choice. A satisfactory nomenclature of these water-soluble vitamins will have to be elaborated.

6. One of these would appear to be the substance known as vitamin B or the antineuritic vitamin. It is rapidly destroyed by alkalis. The other resists treatment with alkali and would seem to be the factor chiefly concerned in the action of the yeast supplements in rendering high-protein diets adequate for growth.

We desire to express our thanks to the Medical Research Council for a grant from which part of the expenses of this research were defrayed. The other part was covered by a grant made to one of us (A. H.) by the Egyptian Educational Mission to whom our thanks are also due.

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XC. A CONDUCTIVITY METHOD FOR THE DETERMINATION OF CARBON DIOXIDE.

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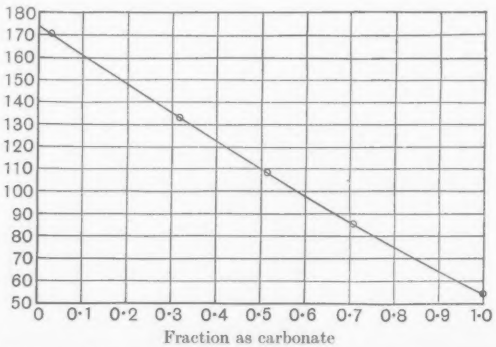
(*Received May 1st, 1927.*)

Being faced with the necessity of absorbing some 100 cc. of CO_2 in 10 minutes and of estimating the quantity absorbed as rapidly as possible and with a possible error not greater than 0·5 %, the usual methods for determining CO_2 were all examined and found wanting in some respect.

It was necessary, therefore, to devise a new and rapid method of estimating carbonate in a mixture of carbonate and hydroxide, and use was made of the fact that the migration velocity of the CO_3'' ion is considerably smaller than that of the OH' ion, so that the conductivity of a solution of NaOH falls as it absorbs CO_2.

Calibration.

This is very easily performed, since it is only a matter of plotting a calibration curve showing the conductivities of mixtures, in varying proportions, of solutions of NaOH and Na_2CO_3 of equivalent concentration. Such a curve is shown in the figure, and it will be seen that it is nearly a straight line for carbonate proportions above 0·3. As points lower than this will practically never be required (the volume of solution used to absorb the CO_2 can be adjusted so as to avoid them) only a few points are required to determine the curve.



Conductivity of mixtures of 0·997 *N* NaOH and 0·997 *N* Na_2CO_3 in varying proportions. Ordinates, equivalent conductivity at 24·5°. Abscissae, proportion of Na_2CO_3 in mixture.

It is not, of course, necessary to use CO_2-free NaOH either for calibrating or for absorbing, since the carbonate content can be determined, and hence the true proportion of carbonate in any mixture of the impure NaOH with pure Na_2CO_3 solution. If c cc. of sodium carbonate solution be mixed with $(1 - c)$ cc. of sodium hydroxide solution containing α equivalents of carbonate per equivalent of total base, then the proportion of carbonate in the mixture is given by

$$\beta = c + \alpha (1 - c).$$

Similarly, if a volume v cc. of CO_2 is absorbed by b cc. of sodium hydroxide solution of total base concentration B, of which a proportion α' is carbonate, then the final proportion of carbonate in the mixture is given by

$$\beta' = \frac{\alpha' Bb + v/11{\cdot}15}{Bb}.$$

Hence $v = (\beta' - \alpha')\, 11{\cdot}15\, Bb$, β' being, of course, determined from the measured conductivity by means of the calibration curve.

As a special case of the above equations, we have the possibility that the solution used for absorbing has the same carbonate content as that used for calibrating, and then it can be seen that

$$v = c\,(1 - \alpha)\, 11{\cdot}15\, Bb.$$

If the method is used for a large number of routine determinations, several samples of caustic soda solution for absorbing will have to be made up; and since it is not easy, or indeed necessary, to ensure that all have exactly the same concentration, a correction will have to be made that will reduce the observed conductivity to that of a NaOH–Na_2CO_3 solution of the same proportion of carbonate as that used for absorbing, but of the total base concentration used in plotting the calibration curve.

An empirical equation has been derived experimentally—by measuring the conductivities of NaOH–Na_2CO_3 mixtures of various concentrations—which holds for concentrations differing from that of the calibrating solution by less than 20 % and for proportions of carbonate greater than 0·3. If the concentration of the solution used for absorbing CO_2 is B/γ (B being that of the solution used for calibrating), λ is the conductivity of the absorbing solution as measured after absorption of CO_2 and λ_0 is the conductivity required, *i.e.* that which the calibrating solution would have if it had the same proportion of carbonate as the absorbing solution, then

$$\lambda_0 = \gamma\lambda \{1 + (1 - \gamma)\, [0{\cdot}15 + 0{\cdot}10\, (\beta - 0{\cdot}03)]\}.$$

In this equation c can be written instead of β if the initial carbonate content of the alkali solutions is small, as it usually is. The values of the constants have been arrived at experimentally, and are subject to an inaccuracy of about 1 %. There will be further errors if very concentrated solutions are used, since the calibration curve will depart too far from the straight line which the analysis assumes.

We can write $\lambda_0 = \gamma\lambda$ with an error of less than 0·5 % when

$1 - \gamma < 0{\cdot}07$ irrespective of sign, for $\beta = 1{\cdot}0$,

$1 - \gamma < 0{\cdot}05$,, ,, $\beta = 0{\cdot}5$.

Having thus determined the proportion of carbonate in the absorbing solution from the calculated value of the conductivity, the volume of CO_2 absorbed is calculated from the equation given above, putting, of course, the value of the concentration of the absorbing solution, not that of the calibrating solution, for *B*.

Technique.

The standard conductivity apparatus is suitable for these measurements, except that, as the equivalent conductivities of the solutions to be used are relatively high, a conductivity cell must be employed which has small electrodes set well apart, so that the actual value of the resistance measured is not less than 10 ohms; trouble may be experienced otherwise, from heating of the solution during measurement, and from the fact that the resistance of the leads to the cell may not be negligible.

The values given on the figure were obtained by means of a metre slide-wire and resistance box as standard resistance, so that only the central portion of the slide-wire need be used. A double commutator and galvanometer were substituted for the usual induction coil and telephones since the measurements had to be made in a noisy laboratory. The position of the contact on the slide-wire could be set to 0·2 mm. so that the error on the resistance measurements was about 0·1 %. It is necessary to keep the temperature of the solution constant to about 0·1°, if readings are to be taken to this accuracy.

SUMMARY.

A method is described of determining the amount of carbonate in a sodium hydroxide-sodium carbonate mixture, by measurement of the conductivity of the solution. This method is chiefly valuable for determining the amount of CO_2 absorbed by a caustic soda solution, and it is believed that it forms the best and quickest method for rapidly absorbing and measuring relatively large quantities of this gas.

XCI. THE POSSIBLE SIGNIFICANCE OF HEXOSE-PHOSPHORIC ESTERS IN OSSIFICATION.

PART VII. THE BONE PHOSPHATASE.

By MARJORIE MARTLAND and ROBERT ROBISON.

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(*Received May 2nd, 1927.*)

In the earlier papers of this series [Robison and Soames, 1924] some account was given of the properties of the phosphatase which is present in ossifying cartilage, bones and teeth, and also in the kidney and intestinal mucosa. In the following we give the results of a more detailed study of some of its properties about which further knowledge was desirable in order to understand the part played by this enzyme and by phosphoric esters in the process of ossification.

Optimum p_H.

It was previously stated that the optimum p_H of this enzyme lies on a flat curve between 8·4 and 9·4, the activity being nearly constant between these limits, but falling off rapidly on either side of them. This statement was based on results of 18-hour experiments, but later observations suggested that the flattening of the curve was in part due to the gradual inactivation of the enzyme at the higher p_H values and that over short periods of time the activity continues to increase with the alkalinity up to a p_H well above 8·4. More exact investigation of the course of hydrolysis has shown that these suppositions are correct. Fig. 1 shows the rate of hydrolysis of glycerophosphoric ester over a period of 5 hours at p_H between 7·25 and 9·4. Further measurements, not shown on the chart, were taken at the end of 24, 48 and 166 hours. The p_H was determined from time to time by a micro-colorimetric method (capillator) on 0·02 cc. liquid withdrawn from the reacting mixture. The addition of indicators to the bulk of the liquid was inadmissible on account of the subsequent colorimetric estimations of inorganic phosphate (Briggs). In this experiment the initial rate increased with the alkalinity up to 9·4, the highest p_H attempted, but at the end of 24 hours the amount hydrolysed at p_H 9·4 (59·8 %) was almost identical with that at 8·9 (59·1 %).

Fig. 2 shows the inactivation of the bone enzyme in 0·4 % solutions of sodium bicarbonate adjusted to different p_H. The rate of inactivation increases rapidly with the p_H above 9·0. At 9·55 over 60 % of the activity was lost in 24 hours.

On the acid side of p_H 6·0 inactivation occurs still more rapidly. In 2 hours at 38° 20 % of the activity was destroyed at p_H 5·2, 50 % at p_H 4·2, and 100 % at p_H 3·2.

In Fig. 3, Curve A, the rate of hydrolysis over a short initial period (1 hour) is plotted against the p_H.

Curve B gives the results of another experiment in which the acid range was more particularly investigated. Here, as in Curve A, a slight increase in the rate of hydrolysis is shown on the acid side of p_H 6·0, and this increase must have been greater but for the partial inactivation of the enzyme during the period of the experiment—in this case 2 hours. Correcting for this loss, Curve B would take approximately the course of the dotted line.

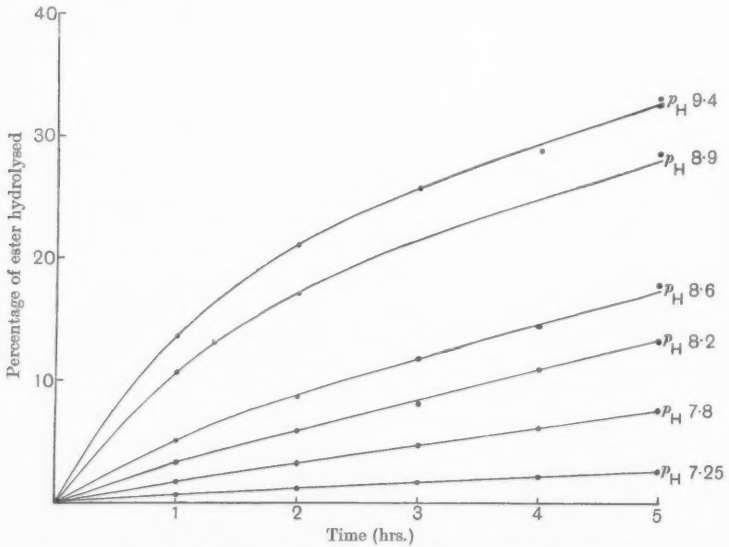

Fig. 1. Hydrolysis of glycerophosphoric ester by bone phosphatase at different p_H. Concentration of ester 0·1 *M*. Temp. 38·0°.

Our intention—to measure the initial velocities of the reaction—has not been completely realised in these experiments for, even in their short duration, the retarding effect of the liberated inorganic phosphate (see below) was appreciable, especially at the higher p_H. The true curve of initial velocities would, therefore, probably show a slightly steeper gradient than that of Curve A.

This activity-p_H curve resembles the dissociation curve of a weak acid, or the dissociation-residue curve of a weak base, and recalls those obtained by Michaelis and his co-workers [1911, 1920] for invertase, except that the slope is in the contrary direction. Michaelis at first explained his results on the assumption that invertase is an acid and that its undissociated molecule

combines with saccharose to form a compound whose decomposition takes place spontaneously as a monomolecular reaction. Subsequent investigation of the relation between the activity-p_H curve and the concentration of the substrate caused him to modify this conception in favour of the hypothesis

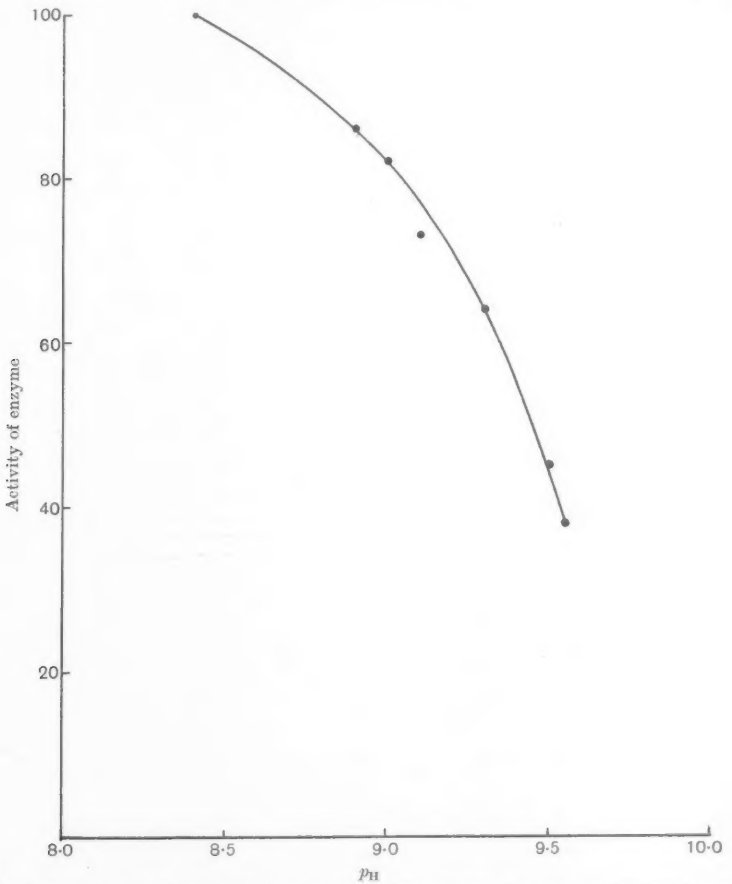

Fig. 2. Inactivation of the bone phosphatase at different p_H. Bone extract kept 24 hours in 0·4 % $NaHCO_3$ solutions adjusted to different p_H. Temp. 38·0°. Activity of enzyme measured by the hydrolysis of 0·1 *M* sodium glycerophosphate in 2½ hours at p_H 9·1, and given as a percentage of the initial activity of the extract.

that invertase combines with the substrate to form a compound with acid properties and that it is the undissociated molecule of this compound which suffers spontaneous decomposition. On this assumption the parameter of the curve is identical with the dissociation constant of the saccharose-invertase acid.

By similar reasoning it may be supposed either (1) that the bone enzyme has the properties of a weak base whose undissociated molecule combines with the phosphoric ester to form a compound which thereupon breaks down into the hydrolysis products of the ester, or (2) that the enzyme combines with the ester to form a compound having the properties of a weak acid, whose anions suffer spontaneous decomposition[1].

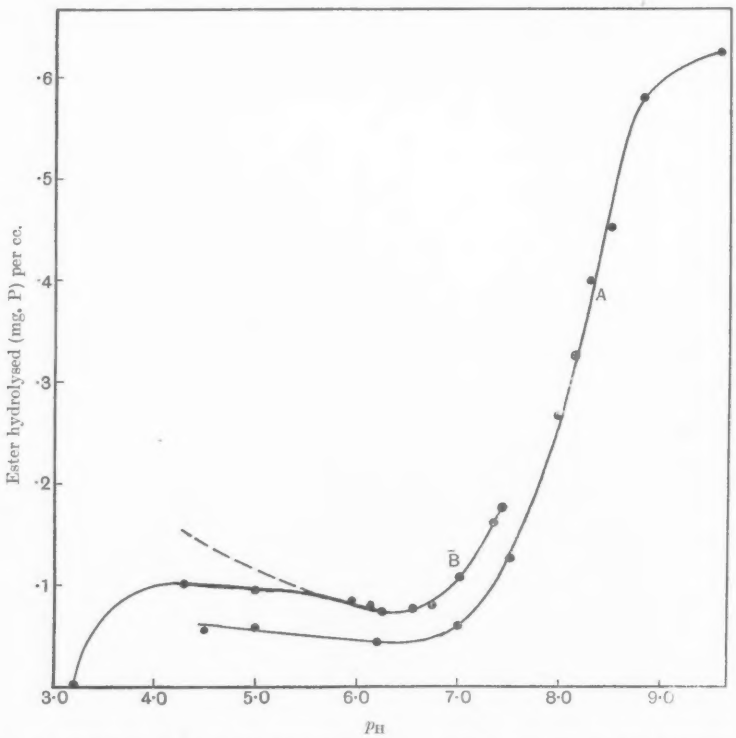

Fig. 3. Activity-p_H curve of the bone phosphatase. Activity measured by the hydrolysis of 0·1 *M* sodium glycerophosphate and given as the amount of ester hydrolysed (mg. P) per cc. in 1 hour. Temp. 39·5°.

Whichever possibility is the correct one, the p_K of this basic or acidic compound would be equal to the p_{OH} or p_H respectively at the point of 50 % dissociation, that is, where the velocity of hydrolysis is half the maximum. The uncertainty connected with the experimental determination of this maximum, which lies in the region where rapid inactivation of the enzyme

[1] There are other possibilities which seem less credible, *e.g.* that the enzyme-substrate complex has the properties of a base, the concentration of whose undissociated molecule governs the rate of the reaction.

occurs, precludes more than a rough guess at the value of this constant. This appears to lie in the neighbourhood of p_H 8·2.

On the second supposition, the acid would thus be weaker than either glycerophosphoric or phosphoric acid, for which the values of p_{K_2} are 6·33 and 6·81 [Meyerhof and Suranyi, 1926].

Our present data are insufficient to decide between these alternatives and some doubt must even be admitted as to whether the interpretation of the graph as a dissociation curve is justified. The possibility of some loss of activity occurring even in short periods at p_H above 9·2 is difficult to eliminate and might account for the flattening of the curve in this region. Further experiments are, however, in progress.

Effect of the concentration of substrate on the rate of reaction.

Michaelis and Menton [1913] found that the rate of hydrolysis of saccharose by invertase was dependent on the concentration of the substrate and that the "activity-p_S" graph, like that showing the relationship between activity and p_H, had the character of a dissociation curve.

We have been unable to demonstrate any corresponding variation of activity of the bone phosphatase with concentrations of ester between 0·003 *M* and 0·3 *M*. Table I gives the results of two experiments lasting 15 minutes and 1 hour respectively. Such small differences as were observed in the amounts hydrolysed are irregular and cannot be attributed to changes in the substrate concentration.

Table I. *Effect of concentration of substrate on the rate of reaction.*

	Amount hydrolysed			
	Exp. 1		*Exp.* 2	
Concentration of ester (glycerophosphate)	p_H 8·2. Temp. 38°. Time 15 mins.		p_H 8·5. Temp. 38°. Time 1 hr.	
	mg. P per cc.	% of ester	mg. P per cc.	% of ester
0·3 *M*	0·041	0·45	0·272	3
0·2 *M*	—	—	0·250	4·5
0·1 *M*	0·039	1·3	0·265	9
0·05 *M*	—	—	0·238	16
0·03 *M*	0·043	4·8	0·274	30
0·01 *M*	0·051	17	0·248	80
0·003 *M*	0·039	43	—	—

Effect of hydrolysis products on the rate of the reaction.

If the values for $k = \frac{1}{t} \log \frac{a}{a-x}$, the velocity constant for a monomolecular reaction, are calculated from the data of hydrolysis experiments such as those quoted on p. 666, these values are found to fall throughout the experiment, and more rapidly than can be accounted for by inactivation of the enzyme. An investigation of the effect of the hydrolysis products on the velocity of the reaction showed that a great part of this retardation is due to the inorganic phosphate, and very little, if any, to the glycerol. Table II gives values of k for the hydrolysis of sodium glycerophosphate with and

without added inorganic phosphate, showing the effect of varying amounts of the latter on the rate of the reaction. With no added inorganic phosphate the values of k fall from 0·0133 to 0·0076 during the hydrolysis of 59·8 % of the ester. The initial value of k is reduced by about 20 % in presence of 0·017 M inorganic phosphate, and by more than 50 % when the concentration of phosphate is increased to 0·033 M. With 0·1 M phosphate the results for the first 5 hours appear to indicate no hydrolysis, and although definite increase of inorganic phosphate was observed after 24 and 48 hours the amount of hydrolysis was less than one-fifth of that found in the control. Results of another experiment with different concentrations of organic and inorganic phosphate are shown graphically in Fig. 4.

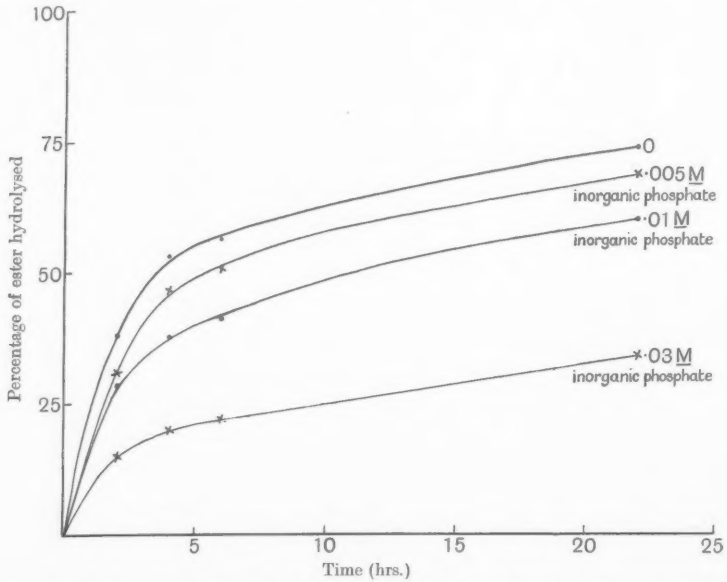

Fig. 4. Effect of inorganic phosphate on the rate of hydrolysis of glycerophosphoric ester by the bone phosphatase. Concentration of ester 0·03 M. Concentration of added inorganic phosphate as shown on each curve. Temp. 38·0°.

Table III gives the results of experiments in which glycerol was added, and shows that this hydrolysis product has very little effect on the rate of the reaction in concentrations up to 0·1 M. The more pronounced effect of glycerol in high concentrations (25 % and over) is discussed in the next section.

The synthetic action of the enzyme.

The depression of the velocity of hydrolysis by inorganic phosphate observed in the previous experiments suggested that the reverse reaction, synthesis of phosphoric ester, was proceeding simultaneously at an appreciable

rate. We were, however, unable to obtain conclusive evidence of synthesis starting from inorganic phosphate and glycerol in the concentrations (0·01 *M*–0·1 *M*) used in these experiments.

Table II. *Effect of inorganic phosphate on the rate of hydrolysis of glycerophosphoric ester by bone phosphatase.*

Concentration of ester 0·1 *M*, p_H 8·05, temp. 37°.
Concentration of added inorganic phosphate as shown below.

	Inorg. P = 0		Inorg. P = 0·017 *M*		Inorg. P = 0·033 *M*		Inorg. P = 0·1 *M*	
Hrs.	% of ester hydrolysed	$k = \frac{1}{t}\log\frac{a}{a-x}$	% of ester hydrolysed	$k = \frac{1}{t}\log\frac{a}{a-x}$	% of ester hydrolysed	$k = \frac{1}{t}\log\frac{a}{a-x}$	% of ester hydrolysed	$k = \frac{1}{t}\log\frac{a}{a-x}$
1	3·02	0·0133	2·48	0·0109	1·28	0·0051	0	0
2	5·67	0·0121	4·62	0·0097	2·67	0·0061	0	0
5	12·11	0·0103	9·17	0·0071	4·98	0·0035	0	0
24	38·97	0·0083	34·05	0·0062	23·58	0·0050	7·73	0·0015
48	59·80	0·0076	50·38	0·0060	32·87	0·0024	11·77	0·0008

Table III. *Effect of glycerol on the rate of hydrolysis of glycerophosphoric ester by bone phosphatase.*

Concentration of ester 0·1 *M*, p_H 8·4, temp. 38°.
Concentration of added glycerol as shown below.

	Percentage of ester hydrolysed		
Hrs.	Glycerol = 0	Glycerol = 0·01 *M*	Glycerol = 0·1 *M*
1	9·7	9·7	9·2
2	15·3	17·5	16·5
5	30·3	31·3	32·2

When the concentration of the glycerol was considerably increased (and the active mass of the water thereby reduced) the synthetic action of the enzyme became very evident, up to 25 % of the inorganic phosphate being converted into phosphoric ester. Synthesis of phosphoric esters was also observed when glycerol was replaced by other polyhydric alcohols, glycol, mannitol, glucose and fructose, but in 50 % ethyl alcohol neither synthesis nor hydrolysis (of glycerophosphate) occurred to an appreciable extent.

The experiments were carried out by adding the alcohols and solid K_2HPO_4 to the bone extract and keeping the mixture at 38°, withdrawing 0·2 cc. into 10 cc. 3 % trichloroacetic acid at intervals. Duplicate determinations of total P and inorganic phosphate were carried out on the protein-free filtrates. No attempt was made to determine the p_H of these concentrated solutions, but the presence of the alkaline phosphate was relied on to maintain a suitable reaction.

The results of some of the experiments are set out in Table IV, the amount of phosphoric ester synthesised being calculated as a percentage of the inorganic phosphate added.

Table IV. *Synthesis of phosphoric esters by the bone phosphatase.*

Alcohol	Concentration of K_2HPO_4 *M*	Amount of P-ester formed calculated as % of inorganic P added				
		1 day	7 days	14 days	21 days	28 days
Glycerol 50 % by vol.	0·07	6·0	22·7	—	24·5	—
„ „	0·2	1·8	12·3	19·3	—	22·6
„ „	0·5	0·8	3·9	5·4	—	10·6
„ 66 %	0·12	—	7·0	12·3	—	16·4
Glycol 25 % by vol.	0·1	3·8	11·9	17·3	—	16·3
„ 50 %	0·1	6·9	17·2	22·4	23·1	—
„ „	0·1	—	18·3	24·8	23·5	—
Alcohol 50 %	0·1	0	0	—	—	—
Mannitol, sat. solution, about 30 %	0·16	—	8·5	9·8	8·3	—
Glucose 60 %	0·12	1·8	2·8	5·8	5·3	—
			3 days			
Fructose 60 %	0·12	—	4·0	—	—	—

The effect of hormones on the rate of hydrolysis and optimum p_H of the enzyme.

In view of the work of Langfeld [1921], who claimed to have demonstrated that the optimum p_H of liver diastase was shifted in presence of various glandular extracts, a series of experiments was performed to find whether such an effect could be obtained with the bone phosphatase. The relative rates of hydrolysis were measured at various p_H ranging from 7·0 to 9·3 with additions of the following hormones.

Adrenaline $\frac{1}{25{,}000} - \frac{1}{2{,}500{,}000}$ (dilution in total volume)

Pituitrin $\frac{1}{25} - \frac{1}{2{,}500}$ („ „)

Thyroid $\frac{1}{400} - \frac{1}{40{,}000}$ („ „)

It is not necessary to give in detail the results of the numerous experiments carried out, but in no case was any definite effect detected on the rate of hydrolysis or the optimum p_H. Similar negative results have also been reported by Demuth [1925].

The action of the bone phosphatase on various phosphoric esters.

Table V shows the action of the bone phosphatase on mono- and diethyl phosphoric esters, which were kindly given to us by Prof. R. H. A. Plimmer and Mr W. J. N. Burch. The enzyme had very little action on the diethyl ester but the monoethyl ester was readily hydrolysed. The enzyme was also found to be without action on a fully methylated derivative of hexosediphosphate prepared by Mr W. T. J. Morgan [1927] whereas the methylhexosidediphosphate was readily hydrolysed. These differences may perhaps be attributed simply to the steric hindrance of the adjacent methyl or ethyl groups, but in view of the much greater affinity of the enzyme for inorganic phosphate than for phosphoric esters, and its very small affinity for glycerol, it is an interesting speculation as to whether the combination of

enzyme and substrate takes place through the hydroxyl groups attached to the phosphorus, the presence of at least two such groups being required.

Table V. *Action of bone phosphatase on mono- and di-ethyl phosphates.*

	Percentage of ester hydrolysed Hours					
Ester	1	2	3	5	24	48
Potassium monoethyl phosphate 0·05 *M*	8·0	11·7	18·9	26	57	69
Potassium diethyl phosphate 0·08 *M*	0·1	0·3	0·3	0·4	0·5	0·7

The discovery of the marked effect of inorganic phosphate on the rate of hydrolysis furnished an explanation of a difficulty encountered in attempting to use the bone phosphatase for the hydrolysis of hexose mono- and di-phosphoric esters and the investigation of their sugar component.

When the ester was used in such concentration as would give a reasonable amount of the sugar, hydrolysis rapidly came to an end, although the enzyme was shown to be still active. The difficulty was overcome by carrying out the hydrolysis in presence of barium ions by which the free phosphate was precipitated in the form of $Ba_3(PO_4)_2$. As the formation of this salt involved a fall in the p_H sufficient baryta was added at frequent intervals to maintain a p_H of about 8·4 to 8·8. In this way quantities of 0·5 g. barium hexose-diphosphate can be almost completely hydrolysed in a few days. The drawback of this method for use with the free hexosephosphoric esters lies in the necessary use of an alkaline reaction and the consequent intramolecular transformation of the sugars which may be anticipated. This objection does not apply to methylhexosides of these hexosephosphates, and the application of this method to these compounds may furnish useful information as to the nature of the sugar [*v.* Morgan, 1927]. The method is in fact analogous to the case of calcification in the body where any increase of inorganic phosphate in the cartilage due to the activity of the enzyme would normally be followed by the precipitation of this excess as calcium phosphate, leaving the activity of the enzyme unimpaired.

Summary and conclusions.

1. The activity of the bone phosphatase increases with the alkalinity up to p_H 9·4, but above 8·4 this is offset by the gradual inactivation of the enzyme.

In the body the optimum p_H may therefore be taken as about 8·4.

2. The activity-p_H curve has the form of the dissociation curve of a weak acid, p_K about 8·2, or the dissociation-residue curve of a weak base.

3. Variations in the concentration of the substrate between 0·003 *M* and 0·3 *M* do not affect the initial rate of hydrolysis.

4. The rate of hydrolysis of glycerophosphoric ester is greatly retarded by inorganic phosphate even in low concentrations, but is not appreciably affected by similar concentrations of the second hydrolysis product—glycerol.

5. In high concentrations of glycerol and in presence of inorganic phosphate, synthesis of phosphoric ester takes place, as much as 25 % of the inorganic phosphate being esterified under certain conditions.

6. The synthetic action of the enzyme has likewise been demonstrated with mannitol, glycol, glucose and fructose.

7. No effect on the rate of hydrolysis or the optimum p_H of the enzyme has been detected as a result of adding adrenaline, pituitrin or thyroid.

8. The action of the enzyme on various types of phosphoric ester is discussed.

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XCII. THE CHEMISTRY OF HEXOSE-DIPHOSPHORIC ACID. PART I.

THE α- AND β- METHYLHEXOSIDEDIPHOSPHORIC ACIDS[1].

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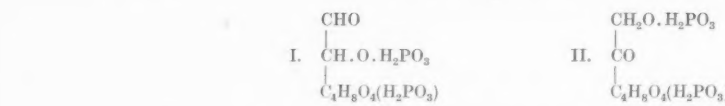
The application of the process of methylation in the sugar group has proved of such great value in the elucidation of the structure of these compounds that it seemed to afford a promising method of investigating the constitution of hexosediphosphoric acid.

The general problem of the chemical constitution of hexosediphosphoric acid divides itself naturally into two parts. The first concerns the exact nature of the hexose portion of the molecule, while the second deals with the position of the two phosphoric acid groups in the six carbon atom chain.

The earlier work of Young [1909] showed that hexosediphosphoric acid on hydrolysis with boiling hydrochloric acid yielded free phosphoric acid along with a laevo-rotatory solution containing fructose or possibly some other reducing sugar. The fact observed by Young that the ratio of the reducing power to the rotation of the product of hydrolysis was greater than corresponds with pure fructose was explained by Neuberg and Kretschmer [1911] as due to the formation of decomposition products owing to the prolonged boiling with acid. That this explanation was not in itself sufficient was shown later by Young who found that pure fructose after heating with phosphoric acid for some hours showed only a slight decrease in the ratio $\frac{\text{Rotation}}{\text{Reduction}}$.

The behaviour of hexosediphosphoric acid towards phenylhydrazine has been investigated both by Lebedev [1909, 1910, 1911] and Young [1911]. The latter worker found that a molecule of phosphoric acid was split off during the formation of the osazone, but that, if the reaction was carried out, using cold reagents, hexosediphosphoric acid reacts without loss of phosphorus to form a derivative which he regarded as a phenylhydrazine salt of hexosediphosphoric acid phenylhydrazone. These reactions led Young [1911] to put forward the following structures which show the probable position of one of the phosphoric acid groups in the molecule. Formula I assumes glucose and formula II fructose as the parent hexose in hexosediphosphoric acid.

[1] This paper was communicated to the Biochemical Society on Feb. 7th, 1927, and an abstract was published in *Chemistry and Industry*, 1927, **46**, 129 (Feb. 11th).



The production of a fully methylated derivative of hexosediphosphoric acid offers a fresh method of attacking the constitution of this substance, since it has been shown by the work of Purdie and Paul [1907], Irvine and Patterson [1922] and Howarth and Hirst [1926] that the methylation and subsequent oxidation of fructose has yielded sufficient evidence for the determination of the constitution of this sugar.

Some early experiments on the production of a methylated hexosediphosphoric acid showed that considerable oxidation occurred during the methylation. When the silver oxide was added to the hexosediphosphoric acid dissolved in methyl alcohol and methyl iodide, a vigorous reaction ensued, but only a small yield of the methylated compound was obtained. Even when the alkylating agent was used in considerable excess, the methyl derivative was produced in very small quantity. As has been explained by Purdie and Irvine [1903] in the case of glucose, the poor yield is partly due to the fact that the alkylation extends to the solvent methyl alcohol and that much of the alkylating agent is thus lost. Examination of the silver residues after the methylation showed that there had been a production of metallic silver, thus indicating that oxidation had occurred. Previous work on the production of tetramethylglucose and tetramethylfructose showed that to avoid oxidation during methylation it was necessary that the aldehydic or ketonic group of the sugar should be protected, and that instead of the free sugar, the methylglucoside or methylfructoside should be used. The application of these principles to hexosediphosphoric acid has led to the preparation of the methylhexosidediphosphates.

Since hexosediphosphoric acid is a derivative of a hexose, most probably fructose, it must necessarily, from stereochemical considerations, exist in α- and β-modifications, corresponding to the α- and β-forms of the parent sugar. Obtained as a result of yeast fermentation, hexosediphosphoric acid has an optical activity, $[\alpha]_D + 3{\cdot}4°$, which suggests that it is a mixture of two isomeric acids. In order to detect the production of the two methylhexosidediphosphoric acids during the condensation of hexosediphosphoric acid with methyl alcohol, this process, and subsequent hydrolysis of the hexoside, were followed by polarimetric observations.

Assuming that hexosediphosphoric acid is a derivative of the ordinary *d*-fructose, the methylhexosidediphosphate corresponding to β-methyl-*d*-fructoside, $[\alpha]_D - 172°$, should possess the greater laevo-rotation of the two hexosides. α-Methylfructoside, $[\alpha]_D - 10°$, is much less laevo-rotatory than the corresponding β-compound, that is, the more strongly dextro- or less laevo-rotatory methylhexosidediphosphate should correspond to α-methylfructoside and would itself be α-methylhexosidediphosphate. Since the methylhexoside-

diphosphate as obtained by Fischer's method is dextro-rotatory, one component of the mixture must possess a dextro-rotation and this, from the above reasoning, would be the α-methylhexosidediphosphate. It has been shown by Purdie and Paul [1907] that α-methylfructoside (β-methylfructoside in their paper) is more rapidly formed than the corresponding β-compound when *d*-fructose is methylated by Fischer's method. Applying these considerations to hexosediphosphoric acid, it would be expected that the α-methylhexosidediphosphate would be formed more rapidly at the commencement of the experiment and thus give rise to an increase in dextro-rotation; the slower production of the β-compound causing the optical rotation of the solution to fall towards the end of the experiment. Exactly the reverse of these optical changes should take place on complete hydrolysis of the mixed methylhexosidediphosphates by acid. There should be a fall in optical rotation, corresponding with the more rapid hydrolysis of the α-compound, followed by a diminished negative rotation towards the end of the hydrolysis. It will be seen from the experimental portion of this paper (Figs. 1 and 2) that there is complete qualitative correspondence between the predicted and experimental changes in optical rotation.

Preparation of barium methylhexosidediphosphate.

The hexoside was prepared from pure barium hexosediphosphate according to Fischer's method [1895]. The methyl alcohol used in all the experiments was free from acetone [see Menzies, 1922] and was fractionally distilled over sodium immediately before use, the portion boiling between 64·5° and 65·5° being collected.

The barium hexosediphosphate (20 g.) was added in the form of a fine powder to 400 cc. of methyl alcohol containing 6·75 g. of dry hydrogen chloride. These quantities give, after complete solution of the barium salt, a solution containing 0·5 % of free hydrogen chloride, which is the concentration of acid used by Fischer throughout his preparations of the α- and β-methylglucosides. In order to obtain the barium hexosediphosphate in solution as rapidly as possible the reaction was carried out in a thick glass bottle containing a number of glass beads which served to grind up any large particles of the salt formed during the rapid addition of the barium salt to the acid methyl alcohol. Under these conditions 5 or 6 minutes' shaking completed the solution of the salt and a clear pale yellow methyl alcohol solution resulted. As soon as this stage was reached a portion of the solution was filtered into a 4 dm. polarimeter tube and the rotation measured. If the precautions mentioned were observed it was possible to obtain a polarimeter reading 12–15 minutes after the barium hexosediphosphate had first come into contact with the acid methyl alcohol. The optical rotation, reducing power and inorganic phosphorus of the solution were determined at intervals until methylation was complete. The values obtained for one particular preparation are shown in Table I.

For the first 2 or 3 hours the solution of the barium hexosediphosphate in acid methyl alcohol remained perfectly clear. The barium chloride after this time was rapidly thrown out of solution as a crystalline mass, only about half of the barium, however, being deposited as chloride during the course of the methylation. During the methylation the temperature was kept at 25° since higher temperatures caused a certain degree of hydrolysis of one of the phosphoric acid groups.

Table I.

Time	Observed* rotation	Total phosphorus g.	Inorganic phosphorus g.	Sugar as glucose per 1 cc. of solution mg.
15 mins.	+0·70°	2·01	0·28	5·29
1 hour 5 mins.	+0·74	—	—	—
4 hours 30 ,,	+0·78	—	—	—
†7 ,, 30 ,,	+0·83	2·16	0·31	2·64
24 ,,	+0·88	—	—	1·80
32 ,,	+0·79	—	—	—
48 ,,	+0·66	—	—	—
56 ,,	+0·57	—	—	—
70 ,,	+0·54	—	—	1·12
78 ,,	+0·54	—	—	—
92 ,,	+0·53	—	—	0·70
100 ,,	+0·53	2·11	0·37	0·51
114 ,,	+0·53	1·96	0·34	0·48
144 ,,	+0·53	—	—	—
168 ,,	+0·525	2·02	0·33	—

* Mercury green line.
† Temperature raised to 25° until methylation was complete.

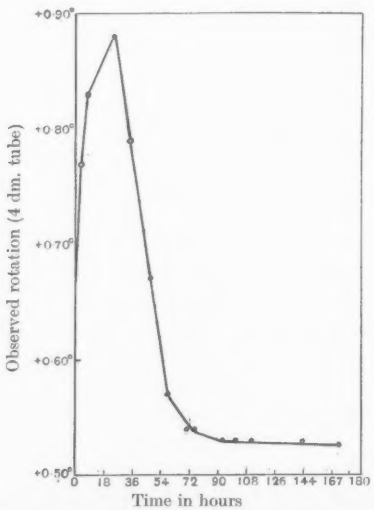


Fig. 1. Curve showing the change in optical rotation during the methylation of hexosediphosphoric acid.

It will be seen from the curve that there is a definite rise followed by a fall in the optical rotation. Since theoretically hexosediphosphoric acid can exist in α- and β-modifications, the rise and fall in optical rotation at once suggest the methylation of two compounds, one of which methylates to give a hexoside with a more strongly positive rotation than barium hexosediphosphate, while, more slowly, a hexoside with a negative rotation is formed. On arresting the reaction after 24 hours, that is, when the maximum positive rotation was obtained, a methylhexosidediphosphate was recovered which possessed a specific rotation of $+ 8{\cdot}3°$ which is more than twice the value of the original barium hexosediphosphate.

Table I shows that during methylation there was a small but definite increase in the inorganic phosphorus showing that slight hydrolysis of one of the phosphoric acid groups of the hexosediphosphoric acid had taken place. In order to be certain that any phosphorus hydrolysed was not converted to methyl phosphate, thus escaping detection as inorganic phosphate, an experiment was made using orthophosphoric acid in methyl alcohol and hydrogen chloride, the conditions of concentration, time and temperature being similar to the main hexoside preparation. No change, however, in the concentration of inorganic phosphate could be detected.

When the hexoside preparation showed no further fall in optical rotation and caused no reduction of Fehling's solution, the barium chloride formed during the methylation was filtered off and the clear methyl alcoholic solution was shaken with a slight excess of barium carbonate in order to remove the excess hydrogen chloride. The solution was again filtered and neutralised to phenolphthalein with a methyl alcoholic solution of baryta. The crude hexoside was precipitated under these conditions as a white amorphous powder.

Separation of the α- and β-barium methylhexosidediphosphates.

Fractionation by alcohol. The crude barium salt, which contained small quantities of barium phosphate, unchanged barium hexosediphosphate and barium chloride, was ground with 5 parts of ice-cold water for about 30 minutes. The mixture was then filtered and the residue again ground with the same quantity of water. This extraction operation was continued until 20 or 25 parts of water had been used. The residue still contained a small amount of organic phosphorus, the last traces of which were exceedingly difficult to remove by extraction with water. The combined filtrates were then fractionally precipitated by the addition of ethyl alcohol.

For the purpose of following the fractionation, the details are given of one particular experiment in which 40 g. of crude, air-dried barium hexosidediphosphate were used.

		Weight of fraction g.
A.	1st fraction. Final alcohol concentration 15 % (by volume)	16·0
B.	2nd ,, Alcohol concentration increased from 15–35 %	7·7
C.	3rd ,, ,, ,, to about 80 %	5·5

The first fraction (A) was dissolved in 10 parts water and increasing quantities of alcohol were added, when the following sub-fractions were obtained:

		$[\alpha]^{18-19°}_{\text{Hg green}}$	Weight of fraction g.
(1)	Fraction precipitated with alcohol up to 5 % concentration	+3·0°	5·0
(2)	" " increasing from 5–10 % conc.	+0·3	5·8
(3)	" " " 10–15 % "	−1·3	1·8
(4)	" " " 15–70 % "	−2·4	1·8

The result of this fractionation showed at once that at least two compounds, one possessing a positive and the other a negative rotation, were present in the crude hexoside preparation.

The second main fraction (B), obtained by increasing the alcohol concentration from 15 to 35 % as shown above, was also dissolved in 10 parts of water and fractionally precipitated with alcohol.

		$[\alpha]^{18-19°}_{\text{Hg green}}$	Weight of fraction g.
(1)	Fraction precipitated with alcohol up to 10 % concentration	−1·1°	3·6
(2)	" " increasing from 10–15 % conc.	−1·9	2·1
(3)	" " " 15–35 % "	Slightly positive	About 1 g.

The third main fraction (C), which was only precipitated by alcohol above 35 % concentration, dissolved readily in 10 parts of 20 % alcohol, thus differing considerably in solubility from the first and second fractions. The process of fractional precipitation was carried out as for the previous two fractions but no indication of separation into two different optically active substances was obtained. The specific rotation of the fractions remained constant at about + 0·7°.

The combined fractions from the first main fraction, which possessed a positive rotation, were again dissolved in 10 parts of water and fractionally precipitated with alcohol, the progress of the separation being followed by the determination of the specific rotation of the various fractions. As a result of several fractionations a barium hexosidediphosphate was obtained which possessed a specific rotation of + 4·6°.

Similar methods of separation were then applied to those fractions which possessed negative rotation, when a barium salt possessing a specific rotation of − 2·3° was obtained.

The occasional occurrence of fractions possessing higher specific rotations for example, values such as − 5·5° or + 5·0°, at once suggested that the resolution into optical isomers was far from complete.

Fractionation by brucine. The optically active fractions obtained were then converted into brucine salts. The barium salt was dissolved in water and a slight excess of the theoretical quantity of sulphuric acid carefully added in the form of a 2 *N* solution. Brucine in 10–20 % excess of the calculated quantity was then added in a warm 50 % alcoholic solution, the mixed solutions possessing a pale yellow colour.

In the case of the brucine salt prepared from the β-methylhexoside-diphosphate crystals separated within a few minutes of mixing the solutions. After standing overnight, the crystals were filtered off, dissolved in 10 parts of hot 50 % alcohol and allowed to crystallise. The process of recrystallisation was carried out several times, the mother-liquors from each recrystallisation being concentrated to about half their original bulk and mixed together.

A portion of the sparingly soluble salt so obtained was dried to a constant weight at 78° and 15–20 mm. pressure over phosphorus pentoxide, no discoloration taking place.

Phosphorus, estimated by modified Briggs-Doisy method	Found P 3·1 %
Calculated for $C_6H_9O_3(PO_4H_2)_2(OCH_3)(C_{23}H_{26}N_2O_4)_4$	P 3·2 %
Specific rotation of the brucine salt in 10 % alcohol (conc. 0·38 %)	$[\alpha]^{18°}_{\text{Hg green}}$ −38·4°

The isolation of the isomeric α-brucine salt in a pure condition proved a much more difficult process. The preparation of the brucine salt was carried out exactly as has been described above for the β-compound. The β-compound present could be slowly removed by concentrating the alcoholic solution of brucine salt and allowing it to stand in the cold room (0°) overnight. The α-compound remained in solution. A micro-crystalline mass of the brucine α-methylhexosidediphosphate could, however, be obtained by pouring this concentrated alcoholic solution into 3 volumes of dry acetone. Under these conditions the excess of brucine remained in solution. This process of acetone precipitation was repeated several times until the brucine salt was free from excess of brucine. It was then fractionally crystallised from hot 96 % alcohol, the process of separation being followed by converting small portions of the fractions into barium salts and determining the specific rotation. The final brucine salt, after drying at 78° (20 mm.) over phosphorus pentoxide, gave the following analytical figures:

Found	P 2·9 %
Calculated for $C_6H_9O_3(PO_4H_2)_2(OCH_3)(C_{23}H_{26}N_2O_4)_4$	P 3·2 %

Barium β-methylhexosidediphosphate.

The brucine β-methylhexosidediphosphate was converted into the barium salt by treatment with a 50 % alcoholic solution of baryta until the mixture was pink to phenolphthalein. The liberated brucine passed into solution while the barium β-methylhexosidediphosphate was precipitated. It was filtered off, washed with warm 96 % alcohol to remove any adhering brucine, and dried in a vacuum desiccator over sulphuric acid. The salt was then dissolved in 10 parts of ice-cold water and filtered to remove any barium carbonate, the barium salt being again precipitated with alcohol and dried. This process of solution in water and reprecipitation with alcohol must be carried out until the barium salt is perfectly free from brucine, otherwise the observed specific rotation will be much more laevo than the true value.

The final barium salt was obtained as a white amorphous substance which was distinctly more soluble in cold than hot water, thus resembling in solubility the original barium hexosediphosphate.

Analysis. For analysis the salt was dried to constant weight over phosphorus pentoxide. After heating for 2 to 3 hours at 100° (20 mm.) it remained perfectly white, in this respect differing from hexose mono- and di-phosphate.

The estimations of methoxyl groups and barium were carried out by Pregl's micro-methods.

	OCH_3	P	Ba
Found	4·93 %	9·76 %	43·75 %
Calculated for $C_6H_9O_3(OCH_3)(PO_4Ba)_2$	4·97 %	9·92 %	43·91 %

Specific rotation of the salt in water (conc. 3·16 %) $[\alpha]^{18°}_{\text{Hg green}}$ −10·4°

The specific rotation of the β-methylhexosidediphosphoric acid (conc. 1·66 %):

$$[\alpha]^{18°}_{\text{Hg green}} -23·2°$$

Molecular weight determination by the cryoscopic method yielded the following results, samples from two distinct preparations being used:

(1) 0·0942 g. barium salt in 9·95 g. water gave Δ = 0·074°, whence apparent molecular weight = 236

(2) 0·0807 g. barium salt in 9·95 g. water gave Δ = 0·053°, whence apparent molecular weight = 284

$C_6H_9O_3(OCH_3)(PO_4Ba)_2$ requires molecular weight = 624; if 100 % dissociated apparent molecular weight = 208

The experimental results are, therefore, consistent with this formula.

The free β-methylhexosidediphosphoric acid, liberated by the addition of the theoretical quantity of sulphuric acid to the pure barium salt, decomposed rapidly. Concentration of the aqueous solution of the acid in a vacuum desiccator led to rapid elimination of the methyl group. By working with ice-cold solutions, it was possible to obtain the acid as a colourless syrup free from inorganic phosphate, but the aqueous solution of this syrup, after standing at room temperature for 36 hours, showed a considerable reducing power, the estimation of which showed that 23 % of the methyl group had been removed.

Barium α-methylhexosidediphosphate.

This salt was prepared by exactly the same method as has been described for the β-compound and was analysed in the same way.

Analysis.

	OCH_3	P	Ba
Found	5·11 %	9·89 %	43·55 %
Calculated for $C_6H_9O_3(OCH_3)(PO_4Ba)_2$	4·97 %	9·92 %	43·91 %

Specific rotation of the salt in water (conc. 3·65 %): $[\alpha]^{18°}_{\text{Hg green}}$ + 8·2°

Specific rotation of the free α-acid (conc. 1·96 %): $[\alpha]^{19·5°}_{\text{Hg green}}$ +19·7°

Molecular weight determination by the cryoscopic method yielded the following results:

(I) 0·0780 g. barium salt in 9·95 g. water gave Δ = 0·062°, whence apparent molecular weight = 235

(II) 0·0557 g. barium salt in 9·95 g. water gave Δ = 0·054°, whence apparent molecular weight = 193

Barium methylhexosidemonophosphate.

During the preparation of the α- and β-methylhexosidediphosphates by Fischer's method, there always occurred a slight increase in the inorganic phosphorus present, thus indicating that hydrolysis of the phosphoric acid groups in the original hexosediphosphoric acid had occurred. The actual amount of inorganic phosphorus so formed varied slightly in each experiment but never exceeded 3·0 % of the total organic phosphorus.

Since one phosphoric acid group is removed by hydrolysis much more readily than the other in hexosediphosphoric acid, it would seem that a hexosemonophosphoric acid, or methylhexosidemonophosphoric acid as it would become after methylation, would be the most likely product. From the crude barium methylhexosidediphosphates a very soluble barium compound was isolated and proved to be a methylhexosidemonophosphate.

As has already been stated (p. 680), fractional precipitation of the barium salt gave no indication of the presence of two optical isomers and fractional crystallisation of the brucine salt from 50 % alcohol also failed to give any separation. The solubility of the salt in 50 % alcohol, however, was such that crystals of the brucine salt could only be obtained from very concentrated solutions. The crystalline brucine salt could, however, be readily obtained from alcohol-acetone solution.

The specific rotation of the brucine salt (conc. 1·45 %)	$[\alpha]^{19°}_{\text{Hg green}}$	−31·7°
Calculated for $C_6H_{10}O_4(PO_4H_2)(OCH_3)(C_{23}H_{26}N_2O_4)_2$	P	2·91 %
Found	P	3·06 %

The brucine salt was converted to barium salt as has been described for the α- and β-methylhexosidediphosphates.

Analysis.

	OCH_3	P	Ba
Found	7·48 %	7·50 %	35·12 %
Calculated for $C_6H_{10}O_4(OCH_3)(PO_4Ba)$	7·57 %	7·57 %	33·52 %

The specific rotation: $[\alpha]^{19·5°}_{\text{Hg green}}$ +0·92 (conc. 1·08 %)

The hydrolysis of α- and β-methylhexosidediphosphate with acid.

Considerable difficulty has been encountered in attempting to remove the methyl group from each of these hexosides while leaving both phosphoric acid groups intact. With complete removal of the methyl group by acid hydrolysis there always occurred a slight removal of the phosphoric acid groups. Under these conditions too rigid a value cannot be placed on the determination of reducing power, as a means of distinguishing the end-point of the elimination of the methyl group. The barium salt of the methylhexosidediphosphoric acid was dissolved in normal hydrochloric acid and kept at 25° until the solution possessed a reducing power which agreed with the complete removal of the methyl group. The optical rotation of the solution was measured at intervals during the hydrolysis, details of which are shown in Tables II and III and Fig. 2.

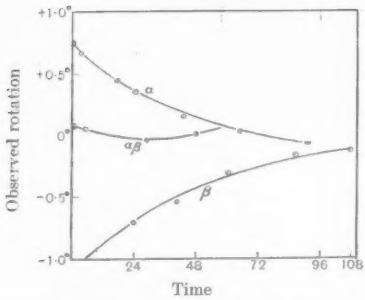


Fig. 2. Optical changes during hydrolysis of α-, β- and $\alpha\beta$-methylhexosidediphosphate with N HCl at 50°.

Table II.

Approximately 0·30 g. of barium β-methylhexosidediphosphate dissolved in 10 cc. of N HCl. Temperature 25°. Rotation for mercury green line.

Time (in hours)	Observed rotation	Total P mg.	Inorganic P mg.	Reducing power as glucose mg.
15 mins.	−1·07°	35	Nil	0·28
2 hours	−1·02	34	—	—
3·5 ,,	−1·00	—	Nil	0·80
24 ,,	−0·70	—	Nil	12·1
40 ,,	−0·54	—	0·5	16·8
62 ,,	−0·30	—	2·1	27·2
86 ,,	−0·19	—	2·8	33·0
110 ,,	−0·15	35	3·4	39·0
134 ,,	−0·08	—	—	—

Table III.

Approximately 0·35 g. of barium α-methylhexosidediphosphate dissolved in 10 cc. of N HCl. Temperature 25°. Rotation for mercury green line.

Time (in hours)	Observed rotation	Total P mg.	Inorganic P mg.	Reducing power as glucose mg.
15 mins.	+0·78°	34·3	Nil	0·19
3 hours	+0·67	—	—	—
18 ,,	+0·57	—	—	13·0
24 ,,	+0·36	35·0	0·5	14·9
42 ,,	+0·14	—	—	21·5
66 ,,	+0·01	35·0	1·4	29·2
90 ,,	−0·08	—	—	41·3

Both α- and β-hexosidediphosphoric acids are produced as a result of the removal of the methyl group but as both forms are unstable in solution, the observed rotation in the case of the α-acid falls while that of the β-acid rises until both values are approximately equal and should, in absence of any hydrolysis of phosphoric acid groups, be equal to the rotation of the commonly known hexosediphosphoric acid in which mutarotation is complete. It will be seen from Tables II and III and Fig. 2, that the final optical value is slightly laevo-rotatory. Presumably this can be explained as due to the production of a small quantity of a laevo-rotatory decomposition product since in both cases a slight production of inorganic phosphate had taken place.

In order to determine the relative rates of hydrolysis of the α- and β-methylhexosidediphosphates, equal quantities of these two substances were

dissolved in the same volume of $N/10$ hydrochloric acid and kept at 50° until the reducing power of each corresponded to complete removal of the methyl group. Estimation of the inorganic phosphate at the end of the experiment showed that not more than 2 % of the total organic phosphate had been hydrolysed. The relative rates of hydrolysis of these two compounds are shown in Fig. 3.

Action of emulsin on the α- and β-methylhexosidediphosphates.

β-Methylfructoside is not hydrolysed by emulsin although this enzyme is generally considered specific in its action on all β-hexosides. Whether emulsin attacks β-methylhexosidediphosphate will, therefore, probably depend on whether or not fructose is the carbohydrate part of the molecule.

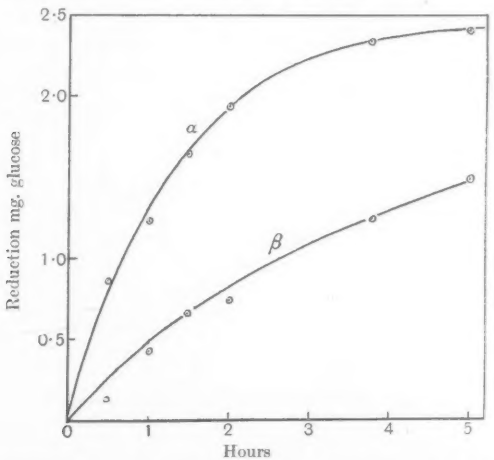


Fig. 3. Hydrolysis of α- and β-methylhexosidediphosphate by $N/10$ HCl at 50°.

Two solutions containing 50 mg. of each of the hexosides in 10 cc. water were treated with 0·2 cc. of concentrated emulsin extract prepared from sweet almonds. The solutions were incubated at 37–38° in the presence of toluene. As a control on the activity of the enzyme preparation, solutions of α-methylglucoside and salicin were treated, under the same conditions, with an equal quantity of enzyme solution. The results are shown in Table IV.

Table IV.

	Reducing power of the solution (mg. glucose) Time in hours					Rotation in 1 dm. tube Time in hours			
	0	3	16	40	64	0	16	40	88
α-Methylhexoside-diphosphate	0·50	0·50	0·48	0·44	0·50	+0·05°	+0·04°	+0·04°	+0·02°
β- "	0·38	0·41	0·38	0·38	0·38	−0·06	−0·07	−0·07	−0·09
α-Methylglucoside	0·62	0·62	0·60	0·55	0·63	—	—	—	—
Salicin	1·36	4·11	14·30	17·20	—	−0·39	−0·24	−0·19	−0·19
Boiled enzyme	0·36	0·36	0·31	—	—	−0·01	−0·01	−0·01	—

Emulsin has, therefore, no hydrolytic action on the methoxyl group of the α- and β-methylhexosidediphosphates. There is, however, a considerable hydrolysis of organic phosphate since the inorganic phosphate present at the end of the experiment was 5·2 % and 7·2 % of the total organic phosphorus for the α- and β-hexosides respectively. That the liberation of this quantity of phosphorus did not in itself rapidly increase the reducing power of the solution showed that the methyl groups remained attached to the reducing groups of the hexosides.

Action of invertase on the α- and β-methylhexosidediphosphates.

50 mg. of each of the hexosides were dissolved in 10 cc. water and treated with 0·5 cc. of invertase solution. The activity of the enzyme was followed by using control solutions of sucrose, α-methylglucoside and salicin. The solutions were kept at 37° throughout the experiment. Complete hydrolysis of the α- or β-compound would yield a reducing solution equivalent to 5·0 mg. glucose. The results of the experiment are shown in Table V.

Table V.

Substance	Reducing power of solutions (as glucose, mg.) Time in hours				
	0	4	20	44	68
α-Methylhexosidediphosphate	1·22	—	2·64	2·64	2·64
β- "	1·25	1·28	1·58	1·59	1·59
α-Methylglucoside	1·25	1·30	1·30	1·62	1·58
Salicin	2·30	2·28	2·34	2·34	2·64
Sucrose	1·27	32·1	58·0	59·0	59·0
Boiled enzyme	1·20	1·30	1·25	1·39	1·39

There has apparently been some hydrolysis in the case of the α-hexoside (25 %). In another experiment 32 % of the α-hexoside was hydrolysed, thus indicating that invertase slowly attacks the α-, but not the β-compound. Removal of the phosphate groups also occurred to the extent of 10 and 13 % of the total organic phosphorus in the α- and β-hexoside respectively. Harding [1912], using a crude invertase preparation, found that the phosphoric acid groups of hexosediphosphate were rapidly removed by hydrolysis.

The hydrolytic removal of the phosphate groups in the experiments described above was no doubt due to hexosephosphatase contained in the invertase, which had been prepared from autolysed yeast.

The methylation of hexosediphosphoric acid.

As has been described in the introduction, a few preliminary experiments were made, during the earlier part of this investigation, on the methylation of the free hexosediphosphoric acid in methyl alcohol solution.

Barium hexosediphosphate (20 g.) in the form of a fine powder was slowly added to 50 cc. ice-cold methyl alcohol containing sufficient sulphuric acid to precipitate the whole of the barium as barium sulphate. After standing for

a few hours in the cold room (0°) the whole of the barium sulphate was precipitated. The clear methyl alcohol solution of the hexosediphosphoric acid was then concentrated to a volume of about 10–15 cc. and methylated according to the method of Purdie and Irvine [1903]. The methylation of the compound was carried out in three stages and a large excess of the methylating agents was used. The methyl alcohol solution was mixed with 12 mols. of methyl iodide and warmed on a water-bath. Silver oxide (6 mols.) was then slowly added over a period of 5 hours and the heating continued for some hours after the last addition of silver oxide. The product was then filtered and the residue of silver oxide and silver iodide extracted with boiling methyl alcohol. The filtrate together with the methyl alcohol extracts was evaporated in a desiccator, when a pale yellow syrup was obtained: yield 2 g. This syrup was again methylated using the minimum quantity of methyl alcohol possible to effect solution (about 5 cc.). A non-reducing colourless syrup was obtained which was free from inorganic phosphate but contained 14·5 % of organically combined phosphorus ($7OCH_3$ requires 14·1 % P). Estimation of methoxyl groups showed that the substance contained 42·5 % as against 49·5 % OCH_3 for the fully methylated hexamethyl-methylhexosidediphosphoric acid. Further methylation produced a small quantity of a colourless syrup which contained 47·8 % OCH_3, thus indicating a mixture of compounds containing 6 and 7 OCH_3 groups. An attempt to distil this syrup under 1 mm. pressure only gave rise to decomposition. Further work on the production of methylated derivatives of hexosediphosphoric acid is in progress.

The hydrolysis of β-methylhexosidediphosphate with bone enzyme.

The discovery by Robison [1923] of an enzyme in ossifying cartilage, which will rapidly remove the phosphoric acid groups of hexosediphosphate by hydrolysis, suggests a further method of attacking the constitution of the hexose portion of the hexosediphosphoric acid molecule. A method of hydrolysing barium hexosediphosphate with the enzyme, the optimum p_H of which is well on the alkaline side of neutrality, is discussed in a recent paper by Martland and Robison [1927] who point out the difficulty of identifying the liberated hexose after it has remained in alkaline solution for several days. The application of this method to β-methylhexosidediphosphate has given more promising results, as the liberated β-methylhexoside does not undergo the Lobry de Bruyn transformation owing to the substituent methyl group attached to the reducing group.

An experiment using 0·3 g. of barium β-methylhexosidediphosphate after treatment with bone enzyme gave, on concentrating the aqueous solution, a syrup which contained 9·9 % OCH_3 (β-methylfructoside requires 15·9 %). A solution of the syrup was non-reducing and strongly laevo-rotatory, $[\alpha]^{18°}_{\text{Hg green}} - 133°$. After boiling a small quantity of the syrup with dilute hydrochloric acid there was a considerable increase in reduction. Control experiments with solutions containing the enzyme together with phosphate

were made and correction made for any optical activity or reducing power due to the enzyme. The few results so far obtained support the evidence of Young and Neuberg that fructose is the hexose part of the hexosediphosphoric acid. Further work on this subject is in progress.

Summary.

1. Two isomeric methylhexosidediphosphoric acids have been prepared from yeast hexosediphosphoric acid.

2. A method is described for the separation of these new compounds.

3. The α-methylhexosidediphosphoric acid is dextro-rotatory

$$[\alpha]^{20°}_{\text{Hg green}} + 19{\cdot}0°,$$

while the isomeric β-compound is strongly laevo-rotatory

$$[\alpha]^{18°}_{\text{Hg green}} - 23{\cdot}0°.$$

4. The barium and brucine salts of both of these compounds are described.

5. The methyl group in α-methylhexosidediphosphoric acid is more rapidly removed by hydrolysis than that in the corresponding β-compound.

6. Neither compound is hydrolysed by emulsin.

7. Invertase causes a partial removal of the methyl group from the α-hexoside. The β-methylhexosidediphosphoric acid is not attacked.

8. The methylation of hexosediphosphoric acid yielded a non-reducing syrup consisting largely of the hexamethyl-methylhexosidediphosphoric acid.

9. Bone enzyme rapidly removes the phosphoric acid groups from β-methylhexosidediphosphate, at the same time producing a strongly laevo-rotatory non-reducing substance possessing the properties of a fructoside.

10. A methylhexosidemonophosphoric acid, which was produced in small quantities during the methylation of hexosediphosphoric acid by Fischer's method, has been isolated in the form of its barium salt.

The author wishes to express his most sincere thanks to Prof. A. Harden, F.R.S., and Dr R. Robison for their constant advice and criticism throughout the whole of this investigation.

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XCIII. THE ANTISCORBUTIC FRACTION OF LEMON JUICE. V.

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It was previously pointed out [Zilva, 1924] that even the purest antiscorbutic fractions possess the property of reducing ammoniacal silver nitrate in the cold and of decolorising potassium permanganate. In view of the fact that the antiscorbutic activity of solutions is destroyed by exposure to oxygen, it was not unreasonable to assume the existence of a possible connection between the two phenomena. The first obvious problem to solve was, therefore, whether the reduction of either or both of the above reagents was brought about by a substance or a grouping in a substance which was also responsible for the antiscorbutic potency. The experiments of Connell and Zilva [1924] pointed to the fact that this was not the case. In that investigation it was shown that although the conditions conducing to the preservation of the vitamin also conduced to the preservation of the reducing properties of an active solution, the destruction of the antiscorbutic activity and of the reducing properties proceeded at different rates.

Owing to the fact that antiscorbutic solutions lose their activity in the presence of atmospheric oxygen it is customary to imply that the destruction of the physiological activity is due to the direct oxidation of the vitamin. Although there is no experimental evidence against this conception one is not justified in excluding the possibility that the deleterious action of the oxidation process may be at least partly indirect, since the purest fractions so far obtained are impure in the chemical sense. In fact, the stability of the active principle may be conditioned by the presence of some of these accompanying "impurities." With the object of throwing some light on this subject the writer has been endeavouring, during the last few years, to modify active solutions in such a way as to destroy their capacity for reducing ammoniacal silver nitrate and at the same time leave the antiscorbutic activity mainly unimpaired. These efforts have not proved successful. Information, however, having a bearing on this problem has, in the meantime, been forthcoming from a different direction.

Phenolindophenol was found to be rapidly reduced in the air to its leucobase by decitrated lemon juice and by active fractions derived from that

source. By using this indicator it is, therefore, possible to estimate the reducing capacity of such preparations titrimetrically. Substances with equal or higher oxidation intensities than phenolindophenol, if present, would, of course, not be accounted for by this method. In this empirical way it is nevertheless possible to obtain definite and consistent data of the reducing capacities of antiscorbutically active fractions under various conditions. An opportunity is thus afforded for correlating the two phenomena.

In this investigation it is proposed to show that the reducing agency (the word agency is used for convenience of expression without implying that a single substance is under discussion), although closely associated with the antiscorbutic factor, is not identical with it. There are, however, indications that its presence, most probably amongst other substances, in active solutions, may contribute to the stability of the antiscorbutic potency.

Experimental.

It was found convenient to employ, in the experiments to be described, a 0·02 % aqueous phenolindophenol solution. This indicator is red in acid and blue in alkaline solution. All titrations were carried out at p_H 7, since acid solutions decolorise the indicator independently of the reducing properties of the solution, whilst in alkaline solutions, as will be shown later, the reducing agency of the antiscorbutic solution deteriorates. The deleterious action of acidity can be demonstrated by adding a little of the indicator to distilled water adjusted to p_H 4·5 and p_H 3·4. After a few hours the colour of the solutions disappears. In the former case the addition of a few drops of ammonia regenerates the colour, in the latter case the colour is irretrievably lost.

Experiments were at first instituted with the object of studying the behaviour of the reducing agency under those conditions under which the behaviour of the antiscorbutic factor has already been established.

The influence of p_H *on the reducing agency.*

Three batches of decitrated lemon juice were adjusted to p_H 5·6, 7 and 9 respectively. Each of the solutions was titrated with the indicator immediately after adjusting the reaction, after 5 hours, and after 22 hours, no precautions having been taken to exclude air. It will be seen from Table I that in the case of the neutral and acid solutions there was comparatively little deterioration of the reducing agency in 22 hours. On the other hand, when the solution was made alkaline almost half of the reducing power was destroyed during the time taken for the adjustment of the reaction (the same batch of decitrated juice was used for the acid experiment). After 2 hours the reducing power was only about one-eighth of that of the original juice and after 22 hours the solution did not reduce the indicator at all. The reducing agency deteriorates, therefore, very quickly in alkaline solution in the presence of air. In this

respect its behaviour is similar to that of the antiscorbutic factor which is very unstable under such conditions [Zilva, 1923].

Table I.

	cc. of indicator per 5 cc. of solution		
p_H	At once	After 5 hours	After 22 hours
5·6	17·1	15	13·7
7	16·5	—	12·0
9	9·4	2	Nil

Influence of aeration on reducing agency.

Decitrated lemon juice which had not been previously precipitated with alcohol was adjusted to p_H 7 and air was aspirated through the solution at room temperature. Samples were removed at various intervals for titration purposes. The original preparation required 18 cc. of the indicator for 5 cc. of solution. After $2\frac{1}{4}$ hours the titre fell to 7 cc.; after 4 hours to 4·9 cc.; after 8 hours to 1·2 cc. No reduction of the indicator could be established after 17 hours. In this case, also, the behaviour of the reducing agency resembles that of the antiscorbutic factor [Zilva, 1922; Daubney and Zilva, 1926]. The decitrated juice at p_H 7·4 aerated for 15 hours was shown to become totally inactivated when tested on guinea-pigs.

Behaviour of the reducing agency in the process of fractionation.

In view of the information available concerning the behaviour of the antiscorbutic factor towards lead acetate as a precipitating reagent, it was of interest to follow the distribution of the reducing agency of decitrated lemon juice in the various fractions brought down by this reagent at different hydrogen ion concentrations. The best part of the vitamin is precipitated by lead acetate within the range of p_H 5·4–7·2. Traces, only, of the factor can be precipitated on the alkaline side of this range, whilst on the acid side, no demonstrable quantities are precipitated at all [Zilva, 1927]. The original decitrated lemon juice, the fraction precipitated by neutral lead acetate at p_H 5·4, and the fraction precipitated at p_H 5·4–7·2 after removal of the first (p_H 5·4) fraction from the solution, were titrated daily during a biological test for a period of about 6 weeks. Table II gives the cc. of indicator taken by equivalents of 5 cc. of each preparation in six representative cases chosen at random. It would appear, firstly, that only a part of the reducing agency is accounted for in the precipitates, secondly, that only about 30–50 % of it is present in the fraction which was shown to contain almost the entire antiscorbutic factor of the original juice (p_H 5·4–7·2), and thirdly, that the totally inactive fraction precipitated by lead acetate at p_H 5·4 shows even a higher reducing capacity than the active fraction. It is, therefore, obvious that the reducing power can have no *direct* connection with the antiscorbutic activity. This conclusion was strengthened by further evidence to be dealt with in a later section.

Table II.

Original decitrated juice	Fraction precipitated at p_H 5·4	Fraction precipitated at p_H 5·4–7·2
21	9·8	5·2
18·7	7·1	4·8
11·1	4·4	4·7
12·5	6·0	4·0
15	6·8	5·0
16·5	5	4·5

Fate of reducing agency in presence of quantities of indicator sufficient to oxidise it only partly.

When phenolindophenol is reduced and the leuco-base allowed to remain in the air, the latter becomes gradually oxidised. It was, therefore, to be assumed that if a quantity of indicator insufficient to oxidise the reducing agency were added, the reduced compound might possibly become oxidised in the air and eventually reduced again. This process would continue until the reducing agency became totally oxidised. In other words, the presence of a small quantity of the indicator could accelerate the oxidation of the reducing agency. This was actually found to be the case. To each of 5 batches of 5 cc. of decitrated lemon juice (not precipitated with alcohol) were added 6 cc. of indicator. 5 cc. of original juice required 13·6 cc. of the indicator and consequently a further 7·6 cc. was required to neutralise the reducing agency in each flask. The solutions, after standing in the air, were titrated at definite intervals. The first was titrated after 60 minutes and required 4·2 cc. of indicator; the second 3 cc. after 90 minutes; the third 2·3 cc. after 100 minutes; the fourth 1·5 cc. after 110 minutes; the fifth 0·6 cc. after 120 minutes. Soon afterwards, a solution treated as above became coloured, *i.e.* the re-oxidised leuco-compound was not again reduced owing to the disappearance of the reducing agency.

Behaviour of the antiscorbutic factor in the absence of the reducing agency.

It is seen from the foregoing experiments that it is possible to remove the reducing agency from antiscorbutic solutions by the addition of phenolindophenol. As this treatment is not drastic there was a likelihood that the antiscorbutic activity would not be destroyed at the same time, especially as it was seen that there is probably no direct connection between the two phenomena. This was actually demonstrated by the following two series of biological tests. In one case the indicator was added to lemon juice, decitrated without the alcohol treatment and adjusted to p_H 7, until it was no longer reduced. In the other case about 14 mg. of phenolindophenol were added per 30 cc. of the decitrated juice. This addition was calculated to oxidise about three-fourths of the reducing agency. Both preparations were then fed to the test guinea-pigs *with the least possible delay*. In both tests, doses of 1·5 cc., 3 cc. and 5 cc. with three animals (250–300 g.) on each dose were employed. Of the series receiving the totally oxidised juice, the three animals

receiving the 1·5 cc. dose died within 43, 49 and 57 days respectively, showing signs of scurvy *post mortem*. All the animals on 3 cc. and 5 cc. doses survived the 60 days of the test period and after being chloroformed were found to be normal at the autopsy. Of the animals receiving the three-fourths oxidised decitrated juice, all, with the exception of one which died of pneumonia after 57 days, survived the 60 days of the test period; only the animals on the 1·5 cc. and 3 cc. doses showed some signs of scurvy at the autopsy. The loss in the antiscorbutic activity in both cases was, therefore, not at all proportionate to the destruction of the reducing agency. These experiments, therefore, prove definitely that there is no direct connection between the two phenomena, and that the antiscorbutic activity persists at least for a short time after the total destruction of the reducing agency in the medium. Attention was consequently directed to the possible function of the reducing agency as a stabilising agency for the antiscorbutic factor and the relative behaviour of the two principles on storage and on heating was studied with this end in view.

The behaviour of the antiscorbutic factor and the reducing agency on storage.

Two sources were employed in this experiment, namely ordinary decitrated lemon juice obtained by removing the acids alone in the way already described in previous communications and alcohol-decitrated juice which was further purified by precipitation with alcohol after decitration and concentration. After the removal of the alcohol in the latter case, the decitrated lemon juice was made up to its original volume with a phosphate buffer solution p_H 6·9. The estimations of the reducing agency and the antiscorbutic potency were carried out in both cases in the original preparation, after 24 hours' storage, and after a week's storage. The storage took place in the cold room, the reaction being kept all the time as nearly as possible neutral. The phenolindophenol titrations took place daily shortly before the administration of the doses to the guinea-pigs during the entire period of testing. As one would expect, there was a certain amount of variation in the titres of corresponding preparations from day to day. Tables III and IV give the maximum, minimum and mean titres for 5 cc. of each of the preparations and a summary of the parallel biological tests. It is to be pointed out that although the differences between the maximum and the minimum titrations were great, most of the figures varied more or less in the neighbourhood of the mean.

The two tests can only be compared in a rough way since the antiscorbutic potency is assessed with much less precision by the biological method than the estimation of the reducing agency by titration with phenolindophenol. The results, however, show that the deterioration of the two principles on storage is more or less of the same order, possibly the loss is greater in the case of the reducing agency. This suggests a possible dependence of the antiscorbutic factor on the reducing agency. Further experiments pointed in the same direction.

Table III.

Ordinary decitrated juice.

	cc. indicator per 5 cc. of solution		
	Original cc.	After 24 hours' storage cc.	After 8 days' storage cc.
Mean	14·4	11·2	9
Maximum	19·9	18·6	15·8
Minimum	11·3	6·5	1·8
Alcohol-decitrated juice.			
Mean	11·5	8·8	5·5
Maximum	17·2	13·0	9·2
Minimum	6·5	5·5	2·8

Table IV.

Description of preparation	Dose cc.	Days alive	Remarks	Autopsy
Ordinary decitrated:				
Original	1·5	60	Chloroformed	Slight scurvy
,,	1·5	60	,,	Normal
,,	1·5	60	,,	,,
After 24 hours' storage	1·5	60	,,	Slight scurvy
,,	1·5	60	,,	,,
,,	1·5	60	,,	Normal
,,	3	60	,,	,,
,,	3	60	,,	,,
,,	3	60	,,	,,
After 8 days' storage	1·5	58	Died	No scurvy
,,	1·5	60	Chloroformed	Slight scurvy
,,	1·5	60	,,	Normal
,,	3	60	,,	,,
,,	3	60	,,	,,
,,	3	60	,,	,,
Alcohol decitrated:				
Original	1·5	58	Chloroformed	Slight scurvy
,,	1·5	58	,,	Normal
,,	1·5	58	,,	Slight scurvy
,,	3	58	,,	Normal
,,	3	58	,,	,,
,,	3	58	,,	,,
After 24 hours' storage	1·5	38	Died	Scurvy
,,	1·5	58	Chloroformed	,,
,,	1·5	53	Died	,,
,,	3	58	Chloroformed	Normal
,,	3	45	Died	Intercurrent disease no scurvy
,,	3	58	Chloroformed	Normal
,,	5	58	,,	,,
,,	5	58	,,	,,
,,	5	58	,,	,,
After 7 days' storage	1·5	40	Died	Scurvy
,,	1·5	33	,,	,,
,,	1·5	38	,,	,,
,,	3	58	Chloroformed	Slight scurvy
,,	3	58	,,	,,
,,	3	38	Died	,,
,,	5	58	Chloroformed	,,
,,	5	37	Died	,,
,,	5	58	Chloroformed	Normal

Influence of heat on the antiscorbutic factor in the absence of air.

In connection with another enquiry now in progress it was necessary to ascertain whether heating antiscorbutic solutions at high temperatures in the strict absence of air destroys their potency to any considerable extent. For this purpose ordinary decitrated lemon juice adjusted to p_H 7 was exhausted in an ampoule under a vacuum pump and washed out with nitrogen which was previously shown by absorption with alkaline pyrogallol to be free from appreciable traces of oxygen. This process was repeated three times and the evacuated ampoule, containing the antiscorbutic solution, was then heated for 1 hour in a steam autoclave under a pressure of one atmosphere and cooled before letting in the air. Such preparations made daily (except during the week-end) were tested for their potency in 1·5 cc., 3 cc. and 5 cc. doses, three guinea-pigs being used on each dose. The loss in the antiscorbutic potency was found to be very small since two of the animals on the lowest dose survived the test period of 58 days; the other one died of an intercurrent disease. All these animals, however, showed signs of scurvy at the autopsy after being chloroformed. The guinea-pigs on the higher doses all survived the test period and only in a few cases very slight indications of scurvy were found *post mortem*. Acidified decitrated lemon juice treated as above and tested out in precisely the same way showed a somewhat smaller loss in the antiscorbutic potency. The control animals receiving the untreated decitrated lemon juice were, of course, fully protected on all the doses. I am indebted to Miss S. M. L. Snelus, F.I.C. for assisting me in the preparation and the testing of these juices.

The behaviour of the antiscorbutic factor and the reducing agency on storage after being heated under anaerobic conditions.

Decitrated lemon juice treated as described in the preceding section showed very little change in its reducing capacity when titrated with phenolindophenol. Experiments with such preparations after storage yielded interesting results. When crude decitrated lemon juice is stored in the cold room for a week there is comparatively little deterioration either in its antiscorbutic activity or in its reducing capacity. Even the minimum daily dose of 1·5 cc. is practically capable of protecting a young guinea-pig from scurvy for 60 days. In the case of the reducing agency there is a loss of about 30 % during this period. When, however, crude decitrated lemon juice at p_H 7 is heated anaerobically under the conditions described above and then stored for a week the loss in both principles is almost complete. Thus three guinea-pigs on a daily dose of 1·5 cc. of the stored preparation succumbed to scurvy within 4 weeks; two animals on a 3 cc. dose behaved similarly, and two animals on a 5 cc. dose had to be chloroformed after 38 days, scurvy being established at the autopsy. The control guinea-pigs receiving the same autoclaved preparation before storing were alive on all the doses after 38 days. The reducing agency

also disappeared almost entirely during the period of storage. A second experiment confirmed the above observation. These results afford one more illustration of the parallel behaviour of the reducing agency and the antiscorbutic factor.

Conclusion.

One of the main results which emerges from this investigation is that the reducing capacity of antiscorbutic solutions from lemon juice as measured by the reduction of phenolindophenol is not directly associated with the activity. This is revealed by the facts, first, that inactive fractions derived by precipitating decitrated lemon juice with neutral lead acetate at p_H 5·4 show greater reducing properties than the active fraction derived from the same source, and second, that by fully destroying the reducing capacity by the addition of phenolindophenol in excess, the antiscorbutic activity of decitrated lemon juice remains almost intact, if tested immediately after treatment. In other words, the antiscorbutic factor, if one views it as a definite oxidisable substance, is of a lower reducing intensity than the reducing agency. It is therefore not surprising that any treatment such as aeration, storage, etc., which destroys the antiscorbutic activity also destroys the reducing power of the solution. Does this reducing property of the lemon juice fulfil a protective function in the sense of a "reduction buffer" or is its presence merely accidental? Admittedly the evidence produced in this communication does not offer a conclusive answer. The study of the antiscorbutic solutions from other sources and of the behaviour on storage of active solutions after treatment with phenolindophenol would go a long way towards elucidating this. Preliminary work on the latter problem shows that there is actually a rapid inactivation of the antiscorbutic potency under these circumstances, but as the results are complicated by other factors the final decision must at present be deferred.

No doubt can, however, be entertained about the result obtained in connection with the behaviour of heated decitrated lemon juice on storage. Definite evidence is produced showing that by heating the solution it was modified in such a way as to render it less stable as regards its antiscorbutic potency. The actual character of this modification is still under investigation and it is hoped that information will be forthcoming which will throw more light on the subject. It is significant that in this case also the reducing agency disappears with the deterioration of the antiscorbutic activity. It would almost appear as if the stability of the antiscorbutic factor depends on a chain of reactions which are kept in equilibrium in the living cell and that on damaging the cell the equilibrium is disturbed and the individual links are progressively damaged with the ultimate inactivation of the vitamin. The presence of some impurities would, according to this view, be a necessary condition for establishing the antiscorbutic potency, since the removal of such stabilising substances would inactivate the vitamin before the preparation

could be tested. The author has been faced by many disconcerting results in the chemical purification of the vitamin which could be explained on these lines. This hypothesis leads to another conception. So far the antiscorbutic factor has been studied mainly from the aspect of a principle the presence of which is necessary in the diet of certain animals in order to prevent the derangement of unknown physiological functions eventually leading to scurvy. Has it a function in the plant? Is it present there as a necessary link in the metabolic cycle? The author has shown that by certain manipulations it is possible to purify the antiscorbutic factor in lemon juice to a very great extent, but the issue is gradually being narrowed down and the direct application of chemical methods is becoming less effective. The results of this investigation suggest that the frontal attack on the problem must now be supported by thrusts in other directions.

SUMMARY.

(1) Decitrated lemon juice and active fractions derived from that source reduce phenolindophenol to its leuco-compound. In this way the reducing capacity of such solutions can be quantitatively determined.

(2) If insufficient of the indicator to destroy the reducing property of such solutions be added, the reduced compound is re-oxidised in the air and is further reduced by the solution. This alternate reduction and oxidation proceeds until the reducing power of the medium is destroyed.

(3) The reducing agency, like the antiscorbutic factor, is destroyed in alkaline medium in the presence of air, on aerating the active solution and on storage. On fractionating decitrated lemon juice it is, however, found in as high quantities in inactive as in active fractions.

(4) On adding phenolindophenol to decitrated lemon juice until the indicator is no longer reduced and testing the treated solution *immediately*, no very appreciable loss in the antiscorbutic activity is observed.

(5) On heating decitrated lemon juice in a neutral or acid medium in an autoclave at a pressure of one atmosphere for one hour, no very appreciable destruction of the antiscorbutic activity or of the reducing capacity of the solution takes place. On storing, however, both functions deteriorate very much more quickly than in untreated decitrated lemon juice.

(6) It is suggested that the stability of the antiscorbutic factor possibly depends on a chain of reactions, which are kept in equilibrium in the living cell.

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XCIV. ON THE COMPOSITE NATURE OF THE WATER-SOLUBLE B VITAMIN[1].

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In 1915 McCollum and Davis [1915, 1, 2] came to the conclusion that a water-soluble accessory factor was necessary in diet in addition to the fat-soluble vitamin already discovered by them [1913]. This conclusion was based upon the results of a long series of experiments upon young growing rats. McCollum and his colleagues also expressed the opinion that there existed two, and only two, such accessory factors in diet, which were named respectively water-soluble B and fat-soluble A [McCollum and Kennedy, 1916]. The water-soluble accessory substance was found present in milk, egg-yolk and wheat embryo, was soluble in alcohol as well as in water, and withstood prolonged heating at the boiling point of water.

The distribution and properties of water-soluble B, as far as this was investigated, were similar to those of the so-called antineuritic or antiberberi vitamin which had been discovered by Eijkman [1897] and his colleagues as the result of their studies upon induced polyneuritis in birds and their clinical observations upon the etiology of human beriberi. McCollum and Kennedy [1916] concluded that the water-soluble B vitamin was identical with the antineuritic vitamin and that "in the production of polyneuritis in birds by exclusive rice feeding or exclusive feeding of a ration made up of purified foodstuffs, the degeneration of the nerve cells is the specific result of a lack of water-soluble B" [1916, p. 501]. This opinion was widely adopted and emphasised by other authorities; for example by Drummond [1917] and by the Medical Research Council in their Report upon the Present State of Knowledge Concerning Accessory Food Factors [1919, pp. 35, 40].

There were, however, several facts already recorded which were incompatible with this view. Schaumann [1911], Cooper [1912] and Chick and Hume [1917, 2] had pointed out that some materials, mostly extracts and concentrates made from various natural foodstuffs, which were potent to cure or prevent polyneuritis, were incapable of maintaining the weight of the

[1] In this paper the term *antineuritic vitamin* signifies the substance necessary in a diet to prevent polyneuritis in birds and paralysis in rats. The term *vitamin B* (Goldberger's Pellagra-preventive, P-P, factor) is confined to the water-soluble vitamin needed to prevent the pellagra-like disorder in rats with lesions of the skin and alimentary tract. The term *water-soluble B vitamin*, or *vitamins* (the water-soluble B dietary factor of McCollum and his colleagues) signifies a combination of the above two vitamins, each of which is necessary to maintain growth and health.

experimental birds, whereas others had both actions. Cooper [1912] emphasised this fact. Working with several naturally occurring foods, he showed that the respective daily doses which must be added to the birds' diet of polished rice to maintain body weight and to prevent polyneuritis respectively, were different and that there was no regular relation between them.

During the last ten years, evidence has been steadily accumulating which is in opposition to the theory that these two vitamins are identical, notwithstanding the fact that their distribution is so similar in naturally occurring foodstuffs. A comprehensive review of the evidence on this point was given in a paper by Mitchell [1919]. More evidence has, however, been added since that date, among others by Heller [1923], Willaman and Olsen [1923], Emmet and Stockholm [1920], and Levene and Muhlfeld [1923].

The evidence against the identity of water-soluble B with the antineuritic vitamin may be summarised under three different headings:

1. *Distribution in nature.* While this is generally the same, there are important quantitative differences. Some materials, for example pure wheat embryo, are rich sources of antineuritic vitamin [Chick and Hume, 1917, 1, 2] but poor in water-soluble B [Osborne and Mendel, 1919, 2]. In the case of milk, meat, green leaves and roots and tubers the reverse is true [Cooper, 1914; Hopkins, 1912, 1920; Osborne and Mendel, 1922; Gibson and Concepcion, 1916; McCollum and Kennedy, 1916; Osborne and Mendel, 1919, 1, 2; 1920; 1922; Chick and Hume, 1917, 3; Vedder and Clark, 1912; Sugiura and Benedict, 1918; McCollum, Simmonds and Parsons, 1918.]

Randoin and Lecoq [1926] have shown that certain yeasts of different origin, while possessing equal power to maintain growth, vary considerably in their antineuritic properties.

2. *Heat stability.* Foodstuffs containing these vitamins can be heated to 100° for 1–2 hours without appreciable loss; at higher temperatures, *e.g.* 120°, the antineuritic vitamin has been found much more sensitive than water-soluble B [Chick and Hume, 1917, 2; Sherman and Grose, 1923; Emmet and Luros, 1920].

3. *Solubility and other properties.* Both principles are soluble in water and can be dialysed. Water-soluble vitamin B is less soluble than the antineuritic vitamin in strong alcohol [Funk, 1912; Cooper, 1913; Kinnersley and Peters, 1925; Drummond, 1917], acetone [Abderhalden, 1920; McCollum and Kennedy, 1916; Steenbock, 1917; McCollum and Simmonds, 1918] and benzene [McCollum and Simmonds, 1918; McCollum and Kennedy, 1916]. Both vitamins can be adsorbed upon animal charcoal or fuller's earth, but adsorption appears to be more complete in case of water-soluble B and recovery more difficult after processes involving precipitation [Drummond, 1917].

Further light has been shed upon the problem recently by the investigations of Goldberger and his colleagues upon pellagra. Those workers have been led to abandon their previous theory that the cause of this disorder is to be traced to an inferior biological value of the protein in the diet. In their

attempts to prevent human pellagra they found that while such a valuable protein as caseinogen (up to 90 g. daily) gave disappointing results, liberal daily supplements of butter-milk (1200 g.), fresh meat (200 g.) or 30–50 g. dried brewer's yeast (and less of certain extracts), were able to cure or prevent pellagra in persons receiving diets on which this disease might confidently be expected to develop [Goldberger and Tanner, 1924, 1925; Goldberger, Wheeler, Lillie and Rogers, 1926]. These foods are all rich sources of water-soluble B and after extending the work to a series of observations on young growing rats [Goldberger, Wheeler, Lillie, and Rogers, 1926; Goldberger and Lillie, 1926] the conclusion has been reached that the results can best be explained by supposing that what McCollum and Kennedy described as water-soluble B vitamin consists of two substances. These are (1) a pellagra-preventive, P-P, factor, and (2) an antineuritic vitamin, both constituents being required to maintain growth and health in the young rat, (1) being less soluble in alcohol than (2) and more resistant to heat. The facts on which this theory is based are, shortly, these. When young rats were maintained on a basal diet deprived of the water-soluble B vitamin but including as much as 30–40 % of yeast heated in an autoclave at 15 lb. pressure for 2½ hours, growth was not maintained and death occurred after a period of 8–10 weeks, preceded in some cases by symptoms of paralysis. If, however, the diet was further supplemented by a small daily dose of an alcoholic extract of corn-meal to provide antineuritic vitamin, the animal grew or remained in normal health even if the dose of autoclaved yeast were much diminished. If, on the other hand, the basal diet was supplemented *only with the antineuritic substance*, growth was not maintained, even when a comparatively large dose was administered. No paralysis occurred but, after a variable period following the arrest of growth, a number of such animals developed a series of symptoms which were considered to resemble human pellagra [Goldberger and Lillie, 1926]. The shortest time taken for development was 7 weeks. The symptoms included ophthalmia, loss of fur and characteristic dermatitis on ears, paws and neck. With the inclusion of a small amount, 6 %, of a preparation made with acidified water from autoclaved yeast, these symptoms cleared up and growth was resumed.

The present experiments confirm the work of Goldberger and his colleagues and support the conclusion drawn by those workers and the other observers already referred to, that McCollum's water-soluble B vitamin and Eijkman's antineuritic substance are not identical.

The work arose out of an examination of the alcoholic extract made from the material adsorbed on charcoal in Peters' [1924] process for purification of the antineuritic vitamin contained in brewer's yeast. The extract had been prepared by Sir C. J. Martin in the hope that it might provide a possible standard for comparison in the titration of foodstuffs for content of the water-soluble B vitamin. Prof. Peters had told us that although the material was highly potent for the cure of polyneuritis induced in pigeons on a diet of

polished rice, he had no experience of its capacity to maintain growth in rats on a diet deprived of the water-soluble vitamin B.

This was investigated, and it was found that the extract would not encourage growth. If, however, supplemented with a daily ration of yeast heated at 120° for 5 hours to destroy the antineuritic vitamin, the rats grew normally and health was maintained.

A series of experiments has been carried out in which yeast and wheat embryo have been taken as sources of the two water-soluble B vitamins. Various extracts and products have been prepared from these and used both alone and in combination, to provide supplementary rations for the basal diet deprived of these vitamins. Observation has been made of the different pathological conditions developed in the rats and a quantitative study made of the substances required for prevention and cure.

Experimental.

The following materials have been prepared and investigated.

Yeast. Whole dried yeast. Fresh brewer's yeast was washed by kneading in ice-cold water to a smooth paste, filtering and pressing, the operation being repeated three or four times. The washed and pressed yeast was dried at 37° for 3 days and ground to a powder in a mill.

Autoclaved yeast. The above dried yeast was heated for 5 hours in an autoclave at 120° and then dried again at 37°. It did not alter in weight but the colour changed from light to dark brown.

Yeast extract [Peters' method, 1924]. The material used was prepared from the extract with acidified 50 % alcohol (alcohol 50 : water 50 : concentrated HCl 1, by volume) of the material adsorbed on animal charcoal after the precipitations by lead acetate and acid mercuric sulphate had been carried out. In our preparation the traces of mercury remaining were removed, before adsorption with animal charcoal, by passing SH_2 through the solution. The precipitation was completed and excess of sulphuric acid removed by adding the appropriate amount of $BaCO_3$, excess being avoided. The acid alcoholic extract was concentrated at a low temperature under reduced pressure and the residue taken up in water. 1 cc. of the final solution administered was equivalent to about 7 g. of the original washed yeast (dry weight).

Wheat embryo. The wheat embryo was a specially pure specimen containing very little bran; it was dried at 37°.

Autoclaved wheat embryo was prepared by autoclaving for 5 hours at 120° and then drying at 37°.

Two extracts were prepared from the wheat embryo.

Absolute alcohol extract. Wheat germ dried at 37° was shaken at room temperature for periods of 5 hours with each of two changes of absolute alcohol. The filtrates were taken almost to dryness under reduced pressure at approximately 40° and the residue shaken up with ether and acidified water. The water-soluble fraction, after being dried and redissolved in water, was tested. It is called the *absolute alcohol extract.*

85 % *alcohol extract.* The wheat germ used in the previous extraction was re-extracted by shaking at room temperature for periods of 5 hours with each of two changes of 85 % alcohol (85 cc. absolute alcohol made up to 100 cc. with distilled water). The combined filtrates were reduced to dryness as before and taken up in an amount of water equivalent to the original weight of wheat germ. This is the 85 % *alcohol extract.*

The following are the details of the experiments. Black and white or albino rats were used and were placed upon the experimental diet when 3–4 weeks old and weighing 40–50 g. They were kept in cages with screened

wire bottoms of ½-inch mesh to prevent contact with the faeces and probable ingestion [see Steenbock, Sell and Nelson, 1923]. The diet was given in a semi-liquid condition in small pots.

The basal diet deprived of water-soluble B vitamins, hereafter called Diet K, consisted of:

Purified caseinogen (specially extracted with ether and alcohol)	21 parts
Rice starch	63 ,,
Cotton-seed oil	11 ,,
Salt mixture[1]	5 ,,

From 0·05 to 0·1 g. cod-liver oil was given to each rat daily according to the size.

1. *Effect on rats of diets lacking in the antineuritic or both water-soluble B vitamins.*

When young rats, 40–50 g. in weight, are placed upon the basal diet K, very slight growth may take place, but the appetite fails and the body weight gradually declines. After 3–4 weeks the body weight is usually about 35 g., the animals are emaciated and present a feeble and miserable appearance. If untreated, they die, usually without any obvious symptoms other than those of inanition.

Table I. *Effect of yeast and wheat embryo—dried, autoclaved or given as extracts —on the cure and prevention of the collapse (so-called "neuritis") occurring in young rats after 3–4 weeks on diet K deprived of water-soluble B vitamins.*

Exp.	Material	Daily dose required for prevention	Daily dose required for cure
A	Yeast, dried	—	0·05 g.
	,, autoclaved	None with 0·2 g.	No cure or alleviation with 0·2 g.
	,, extract (Peters)	—	0·1 cc.*
B	Wheat embryo, dried	—	0·1 g.
	,, autoclaved	None with 0·4 g.	No cure or alleviation with 0·2 g.
	,, extract with absolute alcohol	—	No cure or alleviation with equiv. of 1·0 g. wheat germ
	,, extract with 85 % alcohol	0·2 cc.†	0·3 cc.

* Equivalent to 0·7 g. yeast. † Equivalent to 0·2 g. embryo.

Symptoms of paralysis (see below) were only rarely observed in animals receiving this treatment. Such was also the experience of Drummond, who noted paralytic symptoms in 3 only out of a large number of young rats [Drummond, 1918] on a diet deprived of water-soluble vitamins. Schaumann [1910] observed nervous symptoms in adult rats after 3–4 weeks on a diet of horseflesh heated with alkali to 120° for 1 hour, or of rye bread similarly treated. Hofmeister [1922] has given a detailed study of the paralysis in rats caused by lack of water-soluble vitamins in the diet. He found that the

[1] Salt mixture No. 185 [McCollum, Simmonds and Pitz, 1917].

condition could be best studied when developed in a more chronic form and was of the opinion that when this happened it was probable that the deficiency was not quite complete, otherwise death occurred swiftly and suddenly. In the present experiments with young rats death from inanition usually occurred before there had been time for the development of paralysis. In one series of experiments however (litter 541, Table IV) 5 out of 8 rats in one litter developed symptoms of paralysis within 33 days after receiving the experimental diet. The onset of the paralysis occurred as described by Hofmeister. It was marked by loss of appetite and great restlessness; there was also a progressive loss of co-ordination and of power in the hind legs. The rats swayed from side to side in walking and sometimes would fall down on one side. Later on, the animals maintained a position with the back hunched up and the head bent forward; they became unable to stand upright unless holding on with the forepaws and frequently fell over upon the head. Two animals (rats 29 and 31, Table IV) in this condition were cured with 0·1 cc. of Peters' yeast extract, and three died.

In the majority of cases definite symptoms of paralysis were not observed, and it was at the time of general collapse, occurring after 3–4 weeks upon the experimental diet K, that the effect of curative substances was tested (see Table I). Administration of Peters' antineuritic extract (0·1 cc. daily, equal to 0·7 g. yeast) or the (85 %) alcoholic extract of wheat embryo (0·3 cc. daily, equivalent to 0·3 g. embryo) restored the animal to some degree of health, and weight was maintained for a considerable period, although there was no significant increase These extracts also cured paralytic symptoms promptly in cases where these were observed (rats 29 and 31, Table IV).

The extract from wheat embryo made with absolute alcohol was without effect in relieving the symptoms of paralysis (see rats 28, 32, 33, Table IV). The antineuritic substance in wheat embryo thus appears to be insoluble or only sparingly soluble in absolute alcohol; that present in the extract prepared from dried yeast by the method of Kinnersley and Peters [1925] is stated however to be soluble in absolute alcohol.

If dried yeast or wheat embryo were given in sufficient amount (yeast 0·05 g.; wheat embryo 0·1 g.) the animals were cured, were restored to health and also began to grow. In the autoclaved yeast or autoclaved wheat germ, on the other hand, the antineuritic properties had been destroyed by the strong heating. When these were administered no cures were effected in rats showing paralysis (rat 28, Table IV) or in those suffering from inanition, and the animals died in the same way as those which were untreated.

2. *Development of "pellagra" in rats due to lack of the second of the water-soluble vitamins.*

Young rats which received the basal diet K supplemented with alcoholic extracts made from yeast or wheat embryo did not grow but showed no signs of collapse at 3–4 weeks. After about 6–8 weeks had elapsed they sometimes

died without showing any special external symptoms, but in other cases they gradually developed the condition described by Goldberger and his colleagues (see above) and considered by these workers to resemble human pellagra. The body weight was usually maintained (rat 68, Curve III, Fig. 2), but the animals were in poor condition, they were thin and active, with rough coat and with the

Table II. *Effect of yeast and wheat embryo—dried, autoclaved or given as extracts—on the cure and prevention of "pellagra" occurring in young rats after 6–8 weeks upon diet K, deprived of water-soluble B vitamins, but supplemented by the antineuritic vitamin.*

Exp.	Material	Daily dose required for prevention, g.	Daily dose required for cure, g.
A	Yeast, dried	0·1 + (0·05 –)	—
	,, autoclaved	0·2 + (0·1 –)	0·2
	,, extract (Peters)	None with 0·3 cc.*	—
B	Wheat embryo, dried	0·2	—
	,, autoclaved	0·4	0·4 did not cure
	,, extract with 85 % alcohol	0·8 cc.† delayed onset	—

* Equivalent to 2·1 g. yeast. † Equivalent to 0·8 g. embryo.

In cases where no doses are given marked with the – sign, smaller doses were not tried.

urine often slightly blood stained. Loss of hair took place, and red inflamed patches of skin appeared on the nose and on the backs of the fore feet, which later became oedematous. There was a marked conjunctivitis and in some cases the ears also became red and thickened. The inflamed areas spread and often involved the hind legs and whole areas round the nose and mouth; sometimes the chest became bare and inflamed. The animals survived 2–3 weeks or longer in this condition before death occurred. On *post mortem* examination there were no definite macroscopic signs except a very unhealthy condition of the whole alimentary tract, especially in the small intestine which showed signs of inflammation with atrophy of the mucous membrane and often contained blood-stained mucous.

The condition was also developed in rats which had been saved from collapse on basal diet K by administration of the alcoholic extracts of yeast (rat 12, Curve II, Fig. 1) or of wheat embryo (rat 52, Curve I, Fig. 2). The time elapsing was usually 3–4 weeks after the previous collapse and 6–8 weeks from the beginning of the experiment.

This "pellagrous" condition was cured slowly but surely by addition to the diet of a small daily ration (0·2 g. and upwards) of autoclaved yeast (see Table II A and rat 12, Curve II, Fig. 1), and at the same time growth was restored. Several days, however, usually elapsed before there was a definite improvement. The skin lesions gradually healed, desquamation took place on chest and paws and round nose and mouth; thickened, cheesy layers peeled from the ears, leaving them thin and papery. In about 2 weeks the skin was healed and hair was beginning to grow.

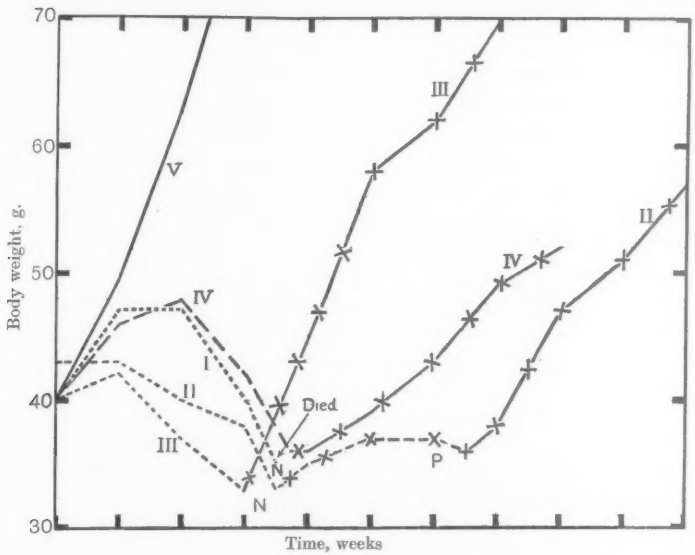

Fig. 1. *Influence of the water-soluble vitamins contained in dried yeast upon the growth of young rats receiving the basal diet K.*

Curve I. Rat 59 ♂ on basal diet without addition. Death at point N due to lack of antineuritic vitamin.

II. Rat 12 ♂ on basal diet without addition for 3½ weeks. Collapse prevented at point N by addition of 0·1–0·3 cc. yeast extract daily (Peters' method). After 6 weeks symptoms of "pellagra" at point P, cured and growth restored by administration of 0·2 g. autoclaved yeast daily.

III. Rat 61 ♀ on basal diet without addition for 3 weeks. Collapse prevented and growth restored at point N by addition of 0·2 cc. yeast extract and 0·3 g. autoclaved yeast daily.

IV. Rat 62 ♀ on basal diet + 0·2 g. autoclaved yeast daily for 3½ weeks. Collapse prevented and growth restored at point N by addition of 0·1 cc. yeast extract daily.

V. Rat 55 ♂ on basal diet + 0·2 g. daily of dried yeast. Normal growth.

Rats 55, 59, 61, 62 were of one litter.

N = Collapse or death due to lack of antineuritic vitamin.

P = Development of skin lesions ("pellagra") due to lack of vitamin B (Goldberger's "pellagra-preventive" factor).

– – – – – – = Basal diet only.

– – – x – – – = Basal diet with daily addition of antineuritic extract of yeast or of wheat germ.

— — — — — = Basal diet with daily addition of autoclaved yeast or wheat germ.

— x — = Basal diet with daily addition of antineuritic extract as well as autoclaved yeast or wheat germ.

——————— = Basal diet with daily addition of whole dried yeast or wheat germ.

3. *Experiments on growth* (*see Table III and Figs.* 1 *and* 2).

Yeast. All symptoms were prevented and some degree of growth was maintained with a very small daily supplement of whole dried yeast (0·1 g.). Some degree of growth was shown also with 0·1 g. of wheat embryo, while normal growth (weekly increase of 10 g. and upwards) was obtained with

0·2–0·4 g. daily of dried yeast or 1·0 g. of wheat embryo (Table III, Exps. A and C).

When the rats received doses of autoclaved yeast up to 0·2 g. daily (rat 62, Curve IV, Fig. 1) there was no evidence of growth, but if supplemented by 0·1 cc. daily of the yeast extract, steady growth took place, although it was subnormal (7 g. a week, Table III B). A normal increase in body weight took place when the rats received 0·4 g. autoclaved yeast together with 0·1 cc. of the yeast extract. Since neither of these materials given alone in similar amount could maintain growth, but could respectively relieve the "pellagrous" and neuritic symptoms, the conclusion is obvious that whole yeast contains both principles, that both are necessary for growth and health, that the antineuritic vitamin is destroyed in the autoclaved yeast and that vitamin B (Goldberger's P-P factor) is absent from this particular yeast extract.

Drummond [1918] found that extracts made from yeast by methods involving precipitation processes had lost the power of promoting growth in rats, whereas a number of other observers, including Peters and ourselves, find similar extracts to be potent antineuritic substances.

Table III. *Effect on growth of young rats of vitamin B, or the "pellagra-preventive" (P-P) factor, in presence of adequate antineuritic vitamin.*

			Given as preventive		Given as cure for collapse due to lack of antineuritic vitamin or of vitamin B	
Experiment	Material	Daily dose, g.	No. of animals	Average weekly increase in body wt. g.	No. of animals	Average weekly increase in body wt. g.
A	Yeast, dried	0·05	—	—	1	1
		0·1	—	—	1	3
		*0·2	1	11	3	9
		0·3	—	—	1	7·5
		0·4	1	23	—	—
B	Yeast, autoclaved (antineuritic vitamin supplied as Peters' yeast extract)	0·1	—	—	1	4
		0·2	1	7	3	5
		*0·3	—	—	2	13
		0·4	1	10	1	10
C	Wheat embryo, dried	0·1	—	—	1	3
		0·2	1	2	3	3
		0·4	—	—	2	1·5
		0·6	1	6	1	6
		*1·0	2	13	—	—
		1·5	1	12	—	—
D	Wheat embryo, autoclaved (antineuritic vitamin supplied as 85% alcoholic extract of wheat embryo or as Peters' yeast extract)	0·2	—	—	1	0
		0·4	1	2	2	4
		0·6	—	—	1	4
		0·8	—	—	2	4
		1·0	1	5	—	—
E	Wheat embryo, dried + 0·3 g. autoclaved yeast	0·2	1	15	—	—
		0·4	2	15	—	
		0·6	1	15	—	

* Dose required for normal growth.

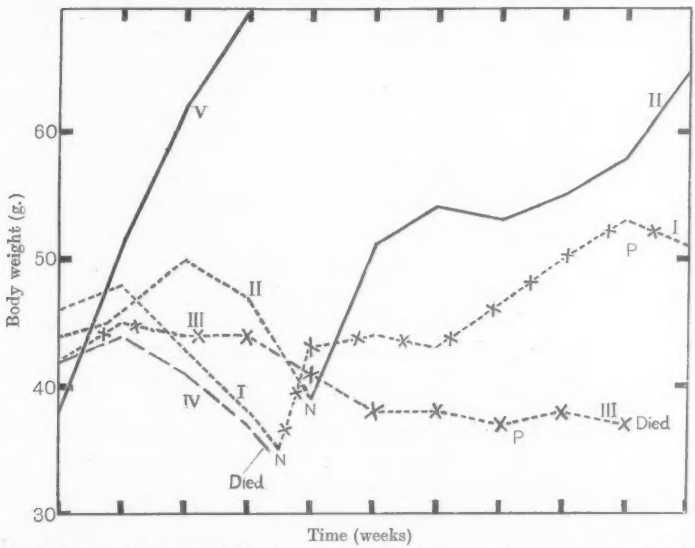

Fig. 2. *Influence of the water-soluble vitamins contained in wheat embryo upon the growth of young rats receiving the basal diet K.*

Curve I. Rat 52 ♀ on basal diet without addition for 3½ weeks. Collapse prevented at point N by addition of 0·7 cc. wheat embryo extract (85 % alcohol) daily. After 9 weeks symptoms of "pellagra" at point P.

II. Rat 30 ♀ on basal diet without addition for 4 weeks. Collapse prevented and growth restored at point N by addition of 0·2 g. wheat embryo daily.

III. Rat 68 ♂ on basal diet + 0·2 cc. wheat embryo extract (85 % alcohol) daily. Symptoms of "pellagra" at point P followed by death 2 weeks later due to lack of vitamin B (Goldberger's P-P).

IV. Rat 67 ♂ on basal diet + 0·4 g. autoclaved wheat embryo daily. Death after 3½ weeks due to lack of antineuritic vitamin.

V. Rat 84 ♀ on basal diet + 1·0 g. wheat embryo daily. Normal growth.

Table IV. *Result of adding wheat embryo and preparations of wheat embryo to diet K of litter* 541*:—*8 *young rats* 27 *days old, of weight* 41–44 *g., of which* 5 *rats developed symptoms of paresis after* 4–5 *weeks on the basal diet.*

					Daily supplements of wheat embryo and preparations given				
		Paresis						Time on basal diet only, before receiving supplement, days	Result
Rat no.	Initial wt. g.	Present	Day of onset	Body wt. at onset, g.	Whole embryo g.	Autoclaved embryo g.	Absolute alcohol extract cc.		
26 ♂	41	0	—	—	—	—	—	—	Died on 26th day
32 ♀	42	+	32	32	—	—	0·2–0·4*	30	„ 35th „
33 ♀	42	+	33	31	—	—	0·2–0·4	30	„ 36th „
28 ♂	43	+	28	30	—	0·2	0·2–0·4	28†	„ 29th „
29 ♂	42	+	33	36	—	0·2 later 0·4	0·2–0·4	30	Cured with 0·1 cc. yeast extract, given from the 36th day
31 ♀	42	+	33	36	—	„	0·2–0·4	30	„ „
27 ♂	42	0	—	—	0·1	—	—	21	Symptoms prevented
30 ♀	44	0	—	—	0·1	—	—	30	„ „

* Equivalent to 3–6 g. of the original wheat embryo.
† Autoclaved wheat embryo given from the 23rd day.

Wheat embryo. The evidence of the existence of the two water-soluble B vitamins in wheat embryo is not quite as dramatic as with yeast. The explanation seems to be that the wheat embryo, if not mixed with other portions of the grain, is comparatively poor in the P-P constituent, while a rich source of the antineuritic factor. Osborne and Mendel [1919, 2], in growth tests with rats, found that commercial wheat embryo, *i.e.* a sample admixed with bran, was a better source of water-soluble B vitamin than a very pure sample of the embryo picked off the seed by hand in the laboratory. The sample of embryo used in the present case was specially pure and contained only a very small proportion of bran or other products. With their commercial wheat embryo Osborne and Mendel obtained good growth with doses less than 0·2 g. daily; with the present specimen 1·0 g. daily was required (see Table III C).

That the P-P factor is present in wheat embryo in very small amount compared with its content of antineuritic vitamin is also evident from the following facts.

(1) When the whole wheat embryo was supplemented with a small daily dose, 0·3 g., of autoclaved yeast, normal growth (an increase of 15 g. weekly) was obtained with a daily dose of only 0·2 g. daily of the embryo (Table III E).

(2) Using the autoclaved embryo, supplemented with antineuritic vitamin from another source, only subnormal growth showing 5 g. increase in weight per week was obtained with a daily dose of 1 g. (Table III D).

(3) The 85 % alcoholic extract of the embryo, which should contain both constituents, if present in the original material, was found to be a good source of the antineuritic vitamin (see Table I B) but to have only a slight effect in preventing the onset of "pellagra" (Table II B).

Discussion.

From the work of others and of ourselves described above, it is clear that the water-soluble B dietary factor of McCollum and his colleagues is not one substance but two at least. McCollum and Kennedy [1916] were evidently mistaken when they adopted the simplest interpretation of the facts at their disposal and pronounced water-soluble B to be identical with the antineuritic substance concerned with the prevention and cure of polyneuritis in birds.

As there are two vitamins two names are required. We would propose to retain the name *antineuritic vitamin* for the antineuritic substance, discovered by Eijkman in 1897 which cured and prevented polyneuritis in birds fed upon a diet of polished rice and was later found to be necessary to prevent marasmus and paresis in mammals.

We suggest that the name *vitamin B* be provisionally confined to the second water-soluble vitamin contained in the dietary factor B discovered by McCollum and Davis in 1915. The substance provisionally called the pellagra-preventive or P-P dietary factor by Goldberger and his colleagues [1926] appears to be vitamin B in this restricted sense. Its presence in a diet is necessary to maintain health in young rats and to prevent decline and death

accompanied by severe skin lesions. Goldberger and his colleagues consider that this condition is the analogue of human pellagra and suggest that deficiency of this vitamin is the cause of the human disease.

As both these water-soluble vitamins are necessary for continued growth and health, it follows that both are present in substances hitherto found to satisfy the requirements of McCollum's water-soluble B, as originally described.

These two vitamins, while both soluble in water, appear (1) to have different solubilities in strong alcohol, benzene and acetone, (2) to possess differing capacities for adsorption on various finely divided solids and (3) to have a markedly different resistance to heat. These differences have made it possible to separate them from one another as they occur in yeast and in wheat embryo, and have provided a method for the study of one apart from the other. On further investigation it may prove that their distribution in nature is by no means as similar as it now appears to be.

In making the above suggestions with regard to nomenclature, we fully realise the incomplete state of our present knowledge concerning the water-soluble B vitamins and the possibility that McCollum's water-soluble B may eventually be shown to contain more constituents than the two dealt with in this paper. The principles responsible for the effects described in experiments of Hartwell [1922, 1, 2], Drummond and Reader [1926], Boas [1927], may or may not prove to be identical with one or other of these two vitamins already differentiated.

Summary.

1. Experiments are described with young growing rats which confirm the views held by many investigators, and recently emphasised by Goldberger and his colleagues, indicating that the water-soluble B vitamin of McCollum and Kennedy contains at least two constituents.

(i) The *antineuritic* (or antiberiberi) vitamin which prevents or cures the collapse, accompanied in some cases with symptoms of paralysis, and leading to death, which occurs in young rats of 40–50 g. weight after 3–4 weeks upon a diet devoid of water-soluble B vitamins. It is identical with the antineuritic substance, discovered by Eijkman in 1897, necessary to prevent polyneuritis in birds fed upon a diet of polished rice.

(ii) The *vitamin B sensu strictu*, identical with the pellagra-preventive or P-P factor of Goldberger and his colleagues. This vitamin can prevent or cure the marasmus accompanied by severe skin lesions, which develops in young rats after 6–8 weeks upon the deficient diet. The effect of its absence can only be demonstrated in cases where the animal is provided with the antineuritic factor in order to prevent the collapse which otherwise would occur at an earlier date.

All known substances containing the water-soluble B vitamin, as defined by McCollum and his colleagues, contain both constituents, since both are necessary for maintenance of growth and health.

2. It is suggested that the term *vitamin B* should be confined to this second water-soluble vitamin (Goldberger's P-P factor), distinct from the antineuritic vitamin, but commonly accompanying it in natural foodstuffs.

3. Yeast is a rich source of both water-soluble B vitamins; pure wheat embryo, while rich in the antineuritic vitamin, was found to be comparatively poor in vitamin B (Goldberger's P-P factor).

4. Vitamin B (P-P factor), as was shown by Goldberger and his colleagues, is much more thermostable than the antineuritic vitamin. A preparation practically free from the antineuritic vitamin can be obtained by heating yeast in an autoclave at 120° C. for 4–5 hours.

5. The strongly antineuritic material extracted from the charcoal in Peters' method of purifying the antineuritic vitamin in yeast was found to be devoid of vitamin B (Goldberger's P-P factor).

We wish to acknowledge our indebtedness to Sir C. J. Martin for suggesting this research to us and also for helping us throughout the course of the work with his constant advice and criticism.

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XCV. THE EFFECT OF DESICCATION UPON THE NUTRITIVE PROPERTIES OF EGG-WHITE.

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In 1922 I was carrying out some experiments upon the calcium and phosphorus retention of rats, for which it was desirable to use a basal diet containing only small amounts of calcium and phosphorus. Egg-white was suggested as a suitable source of protein. Osborne and Mendel [1913, 1, 2; 1915] had shown that young rats could satisfy their nitrogen requirements from a diet in which ovalbumin formed 12 % of the dry weight of the diet and was the sole source of protein, and Bond [1922] had demonstrated satisfactory growth in young rats fed upon a diet containing dried egg-white at a 20 % level as the only protein. For reasons of economy it was decided to use the same source of egg-white as that employed by Bond; this was a dried undenatured preparation of Chinese origin. It was dissolved in water and coagulated by heat before being incorporated in the rest of the diet, for both Bond and Luce had found that the ingestion of raw egg-white by rats was invariably followed by diarrhoea.

The diet, which will be referred to as the ĐW diet, had the following composition:

Dried egg-white	100 g.	Lemon juice	25 cc.
Wheaten starch	250 g.	Marmite	25 g.
Normal salt mixture[1]	25 g.	Distilled water	300 cc.
Hardened cotton seed oil	75 g.		

Each rat received in addition 3 to 5 drops of cod-liver oil daily.

It was found, however, that when 3 weeks old rats were fed on this diet they invariably began to lose weight after a period of about 21 days, and developed a particular pathological condition which is described in detail below, and of which the most striking symptoms were dermatitis, baldness and spastic gait leading on to death.

The symptoms. The rats are as a rule from 21–24 days old when the experiment is started. They grow well and are usually in good health for from 2 to 3 weeks. Then red scaly patches appear at the corners of the mouth, the coat becomes rough and sticky and the long hairs fall out. The fur on the abdomen shows at first a characteristic ribbed appearance, followed by the development of bald areas. Meanwhile the red patches spread to other parts

[1] McCollum, Simmonds and Pitz [1917].

of the body and the picture is one of an eczematous dermatitis. There are even skin haemorrhages in severe cases. The region round the mouth is always the most severely affected, though there is often such marked blepharitis that the eyes are closed. The loss of hair is often extensive. In a few cases oedema of the feet has been seen but this does not usually occur. These rats always have a distinctive somewhat musty smell, probably due to some constitutent of the urine. The body weight remains stationary for a week or two, but falls slowly during the second stage of the disease. This is reached about 2 to 3 weeks after the development of the first signs of deficiency. To the dermatitis, symptoms of nervous upset are now added. There is pronounced spasticity of the limbs, particularly of the hind legs, and the back is arched. The rat assumes in many cases a kangaroo-like posture. The accompanying plate shows the condition at this advanced stage. Some of the rats do not show marked spasticity but assume a crouching attitude and display a curious swimming movement with the front paws. Death, which occurs in the final phase, is preceded by a rapid loss of weight, and the animal shows signs of extreme cyanosis. *Rigor mortis* sets in rapidly. *Post mortem* there is an almost complete absence of fat and the skin is infiltrated and vascularised, but these are the only apparent abnormalities, the organs seeming perfectly normal. No extensive histological study has yet been carried out but sections of liver, kidney, thyroid, spleen and adrenals failed to show any marked changes.

These results were so striking that it appeared that egg-white could not contain proteins satisfactory for complete nutrition and a short note was published [Boas, 1924, 1] to this effect. After this paper appeared, Prof. Mendel wrote suggesting that there must be some important difference between the methods I used and those employed by him, since he had repeatedly found egg-white to be an adequate source of protein for young rats. Shortly after this Dr Helen Mitchell told me that she had used the dried white of hard-boiled eggs with complete success as the sole source of protein for rats [Mitchell, 1925].

The use of fresh egg-white. The experiments were therefore repeated using the fresh white of English or imported eggs instead of the Chinese preparation, and the results showed that I had been too hasty in concluding that egg-white was unsatisfactory as a source of protein. The rats fed upon the fresh egg-white diet grew well and remained in good condition (see Fig. 1). A second note was published [Boas, 1924, 2] withdrawing my former conclusions, and attributing the results obtained to some unknown factor peculiar to the Chinese egg-white.

The question of preservatives. It seemed probable that this unknown factor in the Chinese egg-white might be some toxic preservative. A very thorough investigation carried out in the Government Laboratory by the courtesy of Sir Robert Robertson, however, failed to reveal the presence of any known preservative.

The influence of the nature of the starch. I also communicated with Miss

Bond and learnt that although she had long used the Chinese dried egg-white, yet she had never observed in her rats the symptoms I described. The only qualitative difference between the two diets lay in the nature of the starch, as Bond used potato-starch and I used wheat-starch. This difference did not seem likely to be significant but upon investigation it proved to be of the utmost importance. Rats were fed upon the DW diet until the symptoms were developed to such a degree that death would have been expected in a week or less. They then received a diet similar in all respects to the DW diet except for the presence of potato-starch instead of wheat-starch. There was an immediate increase in weight, new hair appeared and a rapid and lasting cure resulted. Furthermore rats fed on the diet prepared with potato starch from the time of weaning grew well and showed none of the symptoms (Fig. 1). That the wheat-starch was not in itself responsible was clear, for when caseinogen was the protein used the nature of the starch was immaterial.

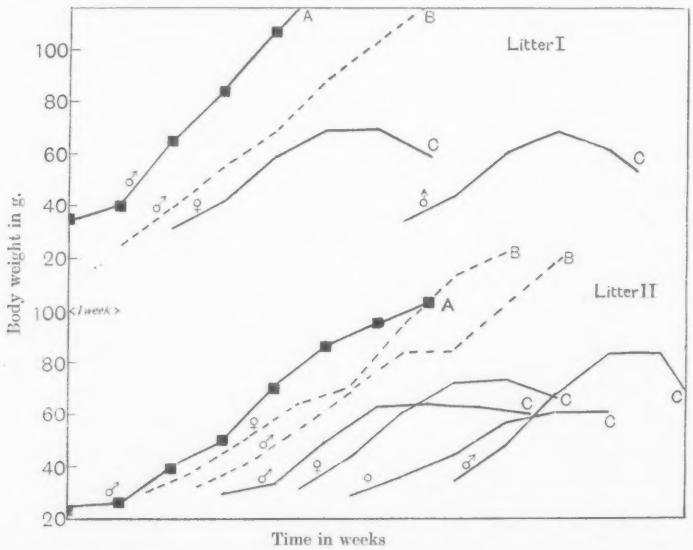

Fig. 1. Curves showing change in body weight of rats receiving diets containing the following different combinations of protein and carbohydrate. A, fresh egg-white and wheat-starch; B, dried egg-white and potato-starch; C, dried egg-white and wheat-starch.

The results of using egg-white dried in the laboratory. The next step was to prepare a sample of dried egg-white in the laboratory. Fresh egg-white was placed in thin layers in flat porcelain dishes in a hot room maintained at 37° and a stream of air blown over it by means of an electric fan for from 12 to 24 hours. In this way a dry flaky undenatured substance was obtained. When this was dissolved in water and used as the protein in the DW diet, exactly the same symptoms resulted as when the Chinese preparation was

used. Thus it was demonstrated that when dried by evaporation at 37°, egg-white suffers some radical change in its nutritive properties which renders it unsuitable for use as the sole protein of the diet of young rats unless potato starch (or some other food substances) are ingested at the same time. Two alternative hypotheses seemed feasible to account for this fact.

(1) The dried egg-white contained some toxic substance which was neutralised by the potato starch.

(2) The dried egg-white had been deprived of some essential dietetic factor which was supplied by the potato-starch and not by the wheat-starch. This could hardly be an amino-acid since the potato-starch was not supplying more than 2 mg. of nitrogen per day (in later experiments protection was obtained when only 0·5 mg. of nitrogen was added daily to the diet by the potato starch).

Whichever hypothesis should prove to be correct the presence of a protective factor in potato-starch must be postulated. This will be referred to as protective factor X.

Experimental details.

The problem seeming one of fundamental interest, a lengthy investigation was undertaken. All the experiments were carried out on litters of rats at the age of 3 weeks, and in all cases control rats from the same litter were included. The date on which the first signs of soreness appeared at the corners of the mouth was noted, and this fact, the weight curve and the general condition of the rats were adopted as criteria of the experiments. In many experiments male rats only were used as they were found to give a quicker and more regular response than the females. Owing to the fact that potato-starch is extremely indigestible to rats, it was found necessary to steam all diets containing more than 20 % of the carbohydrate in this form.

The general results of the experiments are given below. Since experiments involving over 600 rats were carried out, space does not permit them to be described in detail, but curves from representative cases are given (Figs. 1, 2, 3 and 4).

The result of variations in the relative amount of dried egg-white in the diet.

In order to test the first of the two above hypotheses a series of experiments was planned in which diets containing varying concentrations of dried egg-white were used. If a toxic substance were the cause of the symptoms, a decrease in their severity should result from the ingestion of smaller quantities of dried egg-white, while on the other hand an increase in the relative consumption of the egg-white should be followed by the development of a more acute form of the disease. Diets containing approximately 10, 20 and 40 % of their dry weight as dried egg-white were used. The results obtained did not offer support to this theory, for whichever diet was used the condition

was equally severe and appeared as soon. This would however be in accord with the hypothesis that the rats were suffering from a more or less complete deficiency of some essential factor.

The influence of variations in the method of desiccation.

(1) *Reaction.* It was found that the reaction of the egg-white had no influence on the change which took place during desiccation, for if sufficient acetic acid were added to raw egg-white to render it acid to litmus, it still underwent the same change during subsequent dehydration.

(2) *Heat coagulation.* The experiments of Mitchell, referred to above, suggested that if the egg-white were first coagulated by heat, the drying process would be harmless. This proved to be correct, and suggested that the change taking place when egg-white is dried at 37° might be the work of a thermolabile enzyme acting at blood-heat. This idea was however disposed of by the discovery that the egg-white could be exposed in thin layers to the temperature of 37° without undergoing any change in its nutritive properties, provided that desiccation was prevented by the use of wet cloths stretched over the dishes.

(3) *Desiccation* in vacuo. When egg-white is dried *in vacuo* over H_2SO_4 at room temperature the change still takes place, indicating that it is not an oxidation nor the result of exposure to 37°.

(4) *Dehydration by alcohol.* Some egg-white was dehydrated by treatment with successively greater concentrations of alcohol, and it was found that in this case also the egg-white had been damaged. Egg-white dried in this way was denatured during the process.

The protective factor X in potato and other starches and in whole potato.

The protective factor was found present in arrowroot as well as in potato-starch, while the cereal starches—rice and maize—were found to resemble wheat in being devoid of it. Potato-starch seemed to be rich in the factor, for a daily ration of 0·2 g. of raw starch was sufficient to protect rats fed on the DW diet from all the usual symptoms. Raw potato (Fig. 2) proved also a rich source of the factor, protection being given by 1·0 to 1·5 g. equal to 0·2 to 0·3 g. potato-starch per day. Fresh potato juice however was entirely inactive even when large amounts were ingested. The protective factor X therefore is probably so strongly adsorbed as to appear to be insoluble in water. This agrees with the fact mentioned above that raw potato does not contain more of the protective factor than the corresponding amount of potato-starch.

Attempts to deactivate potato-starch by extraction with alcohol and ether were unsuccessful.

The effect of heat and desiccation upon the protective factor X.

Some, though not all, of the protective power of potato is lost when potato is cooked by boiling or steaming. There is also evidence that destruction occurs when potato-starch is mixed with water and steamed. It was mentioned earlier in this communication that the diets containing large amounts of potato-starch were steamed before consumption in order to render them less indigestible. As these diets supplied amounts of the potato-starch far above the necessary minimum, the deleterious effect of heat upon the factor X was not apparent in these cases.

Desiccation at 37° does not appear to damage the protective factor in raw potato to any appreciable extent.

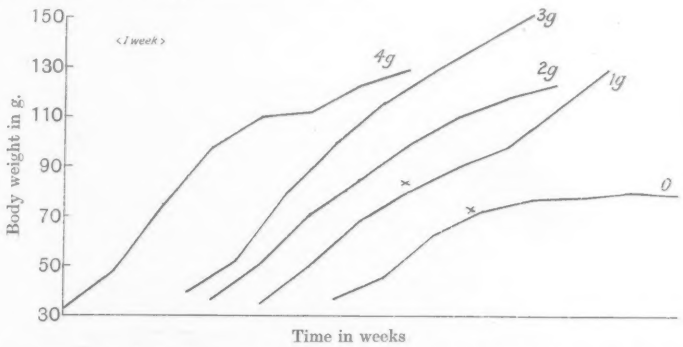

Fig. 2. Curves showing the change in body weight of male rats receiving the DW diet and a daily ration of raw potato. Each curve represents average figures for two or three rats. At × the symptoms of skin lesion appeared.

The question of roughage.

It was noticeable that when rats received potato-starch or raw potato in the diet they produced faeces many times more bulky than those of rats fed upon the egg-white diet prepared with wheat-starch. This bulkiness was due to the presence of undigested starch. If the dried egg-white were supposed to contain some toxic substance, the protective action of the starch might be due simply to mechanical removal of this substance from the intestine before complete absorption could take place. To test this theory agar-agar equal to 10 % of the dry weight of the diet was added to the DW diet. Although by this means bulky faeces were produced, the appearance and course of the usual symptoms were unaffected.

The effect of desiccation upon other proteins.

It was of interest to ascertain whether proteins other than those contained in egg-white suffered a like change when dried. It is quite evident that this is not the case of the vast majority of proteins, seeing that these are commonly dried and preserved in the dry condition before being incorporated in the diet, and can then be used as the sole source of protein

with complete success. In blood-serum the proteins are present in colloidal solution as they are in egg-white, and it therefore seemed possible that if horse-serum were dried in the same way as egg-white it might suffer a similar change in its nutritive properties. This did not prove to be the case. Moreover, the dried serum was found to contain the protective factor.

The effect of supplementing with other proteins.

At this stage in the investigation an incident occurred which, although unfortunate at the time, yet gave information of interest. I was unable to obtain any more Chinese egg-white from the firm which had up to then supplied it. A sample from a different source was tested and found to give the usual results. A larger supply was then obtained from this source and used for a series of experiments, but the results were irregular and unsatisfactory. Although some symptoms of the disease were obtained, in many cases they were only transitory, and followed by spontaneous cures. Only in few cases was the disease at all severe. This egg-white, unlike the first sample, was not entirely soluble in water, as (owing presumably to having been dried at too high a temperature) some of it had become denatured during the drying process. It had already been found that egg-white previously denatured by heat was not damaged by desiccation (p. 716) and the irregular results obtained with this partially denatured specimen suggested that denatured dried egg-white (and presumably fresh egg-white) was able to counteract the deleterious effects of the dried egg-white. If this were also true of fresh egg-white, the facts could be explained by the hypothesis that fresh egg-white contains a dietetic factor not present in the rest of the diet, this factor being destroyed during desiccation, but preserved by heat denaturation.

To test this theory the following experiment was carried out. Six male rats of the same litter were divided into three groups at the age of 21 days. Group A received the ordinary DW diet in which the dried egg-white formed 20 % of the dry weight, and the other groups received the same diet modified in two different ways. In group B the dried egg-white was reduced to 10 % of the dry weight without any addition. In group C the protein of the diet consisted of 10 % dried egg-white and 10 % fresh egg-white.

The curves of increase in body weight of these rats are shown in Fig. 3. In the first two groups the usual symptoms developed but in group C the fresh egg-white was able to give protection. It appeared that this was the minimum amount which would give this result, for after receiving the diet for 3 weeks all the rats showed a slight soreness at the corners of the mouth which lasted for a few days and then disappeared.

The storage of the protective factor X by rats.

In the spring and summer of 1926, the rats receiving the DW diet began to show a greater variability than those of previous work. Some rats or even some whole litters exhibited an unusual resistance to the DW diet. Finally

in September three separate litters which were receiving the DW diet grew well and showed only slight signs of skin affection even after they had received the DW diet for 6 to 7 weeks. There was no variation in the sample of dried egg-white or in the wheat-starch used, and it seemed possible that the rats might in some way have themselves acquired greater reserves of the protective factor than was usual. This idea was based on the assumption that a storage of the factor could take place as has been demonstrated in the case of some of the other accessory factors.

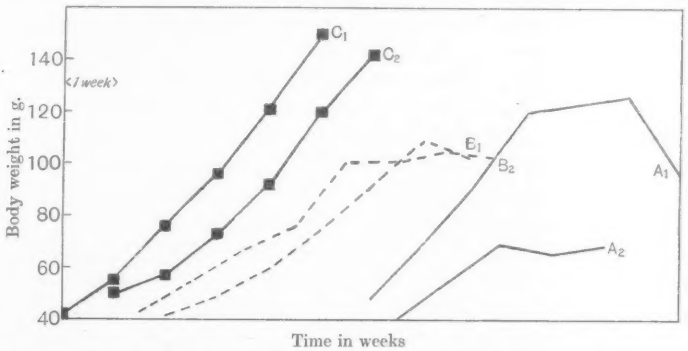

Fig. 3. Curves showing the change in body weight of 6 male rats of the same litter.
Rats A_1 and A_2 received the DW diet.
Rats B_1 and B_2 received the DW diet modified by the reduction of the protein to one-half the usual amount.
Rats C_1 and C_2 received the DW diet modified by the replacement of one-half of the protein by fresh egg-white.

All the rats used had been bred in the Institute on a diet of milk, white and brown bread, marmite, cabbage, carrot, raw meat and various grains. During the past year the amount of water-soluble vitamins in the diet had been increased, in particular by the addition of large amounts of rolled oats and of wheat embryos. It was arranged that some pregnant does should receive a restricted diet during the last week or so of pregnancy and throughout lactation. This diet consisted of white bread, milk, cabbage, carrot and meat. Some litters which did not appear to thrive received a little brown bread occasionally. Although on this diet well-grown healthy litters were obtained it proved a severe strain on the mothers. They were often in such poor condition at the end of the lactation period that they could no longer be used for breeding purposes. I also obtained some litters of rats from an outside source bred on a diet inferior to that of the Institute stock.

Young rats from these two sources were fed upon the DW diet and the results were compared with those obtained with litters the mothers of which had received the full stock diet during lactation. An analysis was made of the condition of the three groups of rats after they had received the experimental diet for 5 weeks. A note was made as to whether the body weight

was rising, falling or stationary and a number of crosses indicated the physical condition of the animal. × indicated no definite symptoms except a rough coat and soreness round the mouth. ×× meant definite dermatitis and loss of hair but unaccompanied by nervous trouble, and when ××× was given both dermatitis and nervous symptoms were severe.

When analysed in this way the results of the experiments showed that the *rapidity* with which the disease developed in young rats was controlled by two things:

(*a*) the weight of the rat at the beginning of the experiment;

(*b*) the diet received by the mother during pregnancy and lactation.

When rats of equal initial body weight were compared, those whose mothers had been fed upon the restricted diet showed a more advanced stage of the disease after they had received the DW diet for 5 weeks than did those from mothers fed upon the usual stock diet.

It appears therefore that rats are able to store the protective factor and that the degree of this reserve in young rats depends not only on their own body weight but also on the amount of the factor in their own previous diet and in the diet of the mother. When possessing large reserves of the factor the young rat can grow well and have an almost normal skin and coat for many weeks on the DW diet, but eventually the reserves are used up and the disease appears and takes its usual course. In an experiment with adult rats of 200–300 g. on the DW diet three months elapsed before the symptoms appeared.

The connection between the protective factor X and the water-soluble vitamins.

The above experiments upon the storage of the factor showed that when the water-soluble vitamin content of the diet was reduced there was an accompanying decrease in the amount of the unknown protective factor. It seemed possible that there might be some connection between this factor and the active principles contained in McCollum's vitamin B, more especially as they possess a similar distribution in many substances, notably egg-white and raw potato. They could not be identical, for potato-starch, one of the most potent sources of the protective factor, is devoid of vitamin B, although from Eijkman's work [1906] it appears to possess some antineuritic power. Further, marmite, which is capable of supplying the water-soluble vitamins, did not contain the protective factor. The work of Goldberger and his co-workers [Goldberger, Waring and Tanner, 1923; Goldberger and Tanner, 1924, 1925; Goldberger, Wheeler and Tanner, 1925; Goldberger, Wheeler, Lillie and Rogers, 1926; Goldberger and Lillie, 1926] was of great interest in this connection. Most of the work was done on cases of human pellagra. Goldberger demonstrated that water-soluble B consisted of two different factors, one the antineuritic or antiberiberi, and the other the pellagra-preventive. He found that the first was more easily destroyed by heat than was the second, for

yeast autoclaved at 120° for 5 to 6 hours was found to have retained its curative power towards pellagra, although the antineuritic potency was lost. Although often found together in nature, the two factors did not show an identical distribution, wheat germ for example being much richer in the antineuritic factor than in the pellagra-preventive.

The rats fed upon the DW diet showed symptoms which in many ways resembled those of pellagra, and it seemed of interest to determine the content of the protective factor X in those substances which Goldberger had found to be rich in his pellagra-preventive factor. Table I shows roughly the content of the protective factor X and of the supposed two constituents of the water-soluble vitamins in a number of foodstuffs. It will be seen that all three are present in dried yeast (Fig. 4); when the yeast is heated for a long time at a high temperature (4 to 5 hours at 120°) the antineuritic vitamin is destroyed but the other two are unaffected. Milk also contains all three, which are therefore found as impurities in commercial caseinogen. Extraction of the caseinogen with alcohol removes the B vitamins but not the protective factor X.

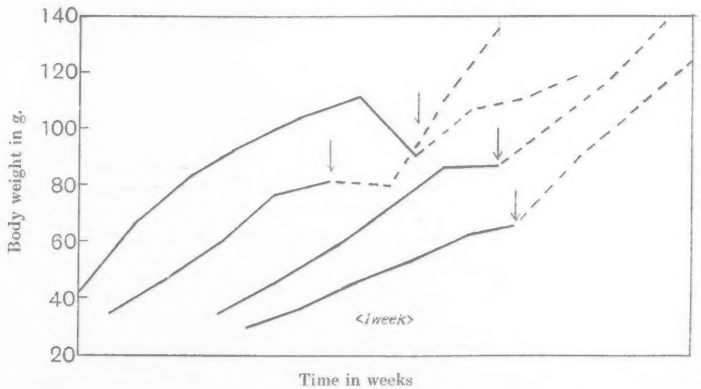

Fig. 4. Curves showing the change in body weight of rats fed upon the DW diet. At the arrows daily rations of dried yeast (0·2 to 0·4 g.) were given in addition.

Raw potato contains both the antineuritic vitamin and the protective factor (it has not yet been tested for the pellagra-preventive). The protective factor remains associated with the starch throughout its preparation, while the antineuritic is present in potato juice and is washed out of the starch.

Thus it appears that the protective factor X, though having in many ways a similar distribution to vitamin B, does not coincide with either of its supposed constituents. This is confirmed by the nature of the symptoms produced when the protective factor is absent from the DW diet. The nervous symptoms (spasticity of the limbs) are not the same as the paralysis induced in rats by deprivation of the antineuritic factor, nor are the skin lesions identical with

those observed in cases of experimental pellagra in rats as described by Goldberger [Goldberger and Lillie, 1926] and confirmed by Chick and Roscoe [1927]. Rats deprived of the pellagra-preventive factor by these workers developed scaly thickened ears and inflamed oedematous feet, whereas the rats in my experiments never showed the first and only in a few cases the second.

Table I.

Showing the distribution in certain substances of the two constituents of vitamin B and of the protective factor X. The figures for the antineuritic vitamin are taken from the Medical Research Council's Report on the Present State of Knowledge of Accessory Food Factors [1924] and those for the pellagra-preventive factor from Goldberger's work and from the work of Chittenden and Underhill [1917] and Underhill and Mendel [1925] on "black-tongue," a pellagrous-like condition in dogs.

Substance	Protective factor X	Water-soluble B of McCollum: Eijkman's anti-neuritic	Water-soluble B of McCollum: Goldberger's pellagra-preventive
Raw potato	+ + +	+ +	
Cooked potato	+	+	
Dried potato	+ +	+ +	
Potato-starch	+ + +	+ (Eijkman)	
Cooked potato-starch ...	+		
Potato-juice	0	+ +	
Arrowroot	+ + +	0	
Cereal starches	0	0	0
Dried yeast	+ + +	+ + +	+ + +
Autoclaved yeast	+ +	0	+ +
Marmite	0	+ +	+ +
Harris vitamin B concentrate	?	+	+ +
Fresh egg-white	+	0	
Dried egg-white	0	0	
Dried coagulated egg-white	+	0	
Dried egg-yolk	+ +	+	
Fresh milk	+	+	+
Commercial caseinogen ...	+	+ +	+
Crude lactalbumin	+	+	+
Extracted heated caseinogen	+	0	0
Dried horse-serum	+		
Wheat-germ	?	+ +	+
Wheat-bran	0	+	
Wheat-flour	0	0	0
Wheat-starch	0	0	0
Wheat-gluten	+		
Malt-extract	0	+	
Raw meat	0	+	+ +
Fresh spinach	+		
Fresh cabbage	+	+	
Banana	+	+	
Butter	0	0	+ Black tongue

Discussion.

In the earlier stages of the investigation it seemed possible that the phenomena observed might be explained by one of the following two alternative hypotheses.

(1) When fresh crude egg-white is dried an essential dietetic factor in it, the existence of which has been hitherto unrecognised, is destroyed. In the absence of this factor from a diet in which the nitrogen is entirely supplied by the egg-white and is complete in all other respects young rats develop a

characteristic universal dermatitis accompanied by nervous disorder which is finally terminated by death. This factor X is present in fresh eggs, both in the yolk and in the white, in dried yeast, raw potato, potato-starch and arrowroot, milk, blood-serum, banana and in fresh spinach and cabbage leaves.

(2) Some toxic product formed when fresh egg-white is dried is responsible for the symptoms which develop when rats are fed on a diet containing dried egg-white as the sole source of protein. The toxic substance can be neutralised by a factor X present in the foodstuffs enumerated in hypothesis (1).

The investigation has not yet reached a stage at which it is possible to decide which, if either, of the above hypotheses is correct. Neither seems entirely satisfactory. So far as at present investigated, egg-white is the only crude protein mixture which is susceptible to this deterioration of its nutritive properties on drying. Dried horse-serum, commercial preparations of caseinogen, gluten and lactalbumin, when they were the only proteins supplied, did not produce the symptoms which were shown by rats receiving a diet in which dried egg-white was the sole protein.

Many facts of the problem which appear irreconcilable may ultimately prove to be due to differences in the amount of the factor X present. Suppose egg-white to contain only just sufficient of it, then a partial destruction of the factor during desiccation would result in the appearance of the symptoms, whereas caseinogen, serum, etc., might contain so much that the loss of a part of it would still leave sufficient for the needs of the animal.

Experiments now in progress however indicate that there is a relationship between the amount of dried egg-white ingested and the amount of the protective factor X needed to give protection.

It seems therefore probable that the true explanation of the problem is a more complicated one than either of the two above theories. It may have some relation to the problem studied by Hartwell [1924, 1, 2; 1925] and also recently by Reader and Drummond [1926]. They find that a dietary factor having a similar distribution to vitamin B was able to correct a diet overbalanced by excess of protein. Since marmite contains this factor, it cannot be the same as the protective factor X, but it is quite conceivable that in the problem set out in this paper a similar question of balance between two constituents of the diet is involved.

SUMMARY.

(1) A diet of crude egg-white, boiled and supplemented with wheat-starch, cotton-seed oil, cod-liver oil, lemon juice, marmite, salts and water, supports young rats in growth and health.

(2) If the egg-white is previously dried the diet is inadequate unless the carbohydrate is supplied in the form of potato-starch or arrowroot, or unless certain substances are added in small amounts.

(3) The condition which is developed by rats fed on this unsatisfactory diet is described in detail.

(4) The change which takes place in dried egg-white is independent of the reaction of the solution during drying.

(5) It is not the work of a thermolabile enzyme.

(6) It does not appear to be a process of oxidation.

(7) Egg-white previously coagulated by boiling is not damaged by desiccation.

(8) The crude proteins of horse-serum and milk do not suffer a similar change during desiccation.

(9) The foodstuffs which possess the power of counteracting the ill-effects resulting from the ingestion of the dried egg-white are raw potato, potato-starch, arrowroot, dried yeast, fresh egg-white, egg-yolk, milk, commercial caseinogen, crude lactalbumin, spinach and cabbage leaves, banana, and dried horse-serum. The presence of a protective factor X in these substances is postulated.

(10) This factor shows a similar distribution in many ways to that of the water-soluble B vitamins. It is not however identical with either the anti-neuritic factor or Goldberger's pellagra-preventive.

(11) Its resistance towards heat and desiccation varies according to the substance in which it is found.

(12) The resistance shown by rats to the ill-effects of the dried egg-white diet depends chiefly on the body weight of the animal at the beginning of the experiment. It is also influenced by the diet of the mother during pregnancy and lactation. It seems probable therefore that rats can store reserves of the protective factor X.

I should like to record my gratitude to Sir C. J. Martin and Dr H. Chick for their unfailing help and advice throughout the course of this investigation.

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UNIV. OF MICH.

XCVI. A NOTE ON THE VITAMIN D CONTENT OF THE STOMACH OIL OF THE AUSTRALASIAN PETREL (*AESTRALATA LESSONI*).

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(*Received May 2nd, 1927.*)

The Australasian petrel (*Aestralata lessoni*), or mutton bird, flies North from the antarctic regions during the summer months, to the islands of the South Pacific, where mating and breeding take place. Here its staple diet consists of "whale-feed," vast shoals of which are met with throughout the temperate seas of the southern hemisphere.

This "whale-feed," according to G. M. and G. S. Thomson [1923] consists largely of a bright red shrimp, which is a swimming stage in the life-history of the crustacean *Munida gregaria*. Carter and Malcolm [1926] state that this crustacean is also the principal food of the Australasian red cod at this time of the year.

Mutton bird oil is obtained from the stomach of the young petrel, and is commonly believed to be supplied to the young from the stomachs of the parent birds. It is stated by Carter [1921] to consist for the most part of cetyl oleate and other esters of cetyl alcohol. As, however, Carter and Malcolm [1926] found no trace of cetyl alcohol in the oil extracted from "whale-feed," it is obvious that mutton bird oil is not the fatty residue from the digestion of the latter. Its origin is mysterious. Rosenheim and Webster [1927] suggest that it is a special secretion used for oiling the feathers by the agency of the bird's beak. Comparative growth tests have been made on this oil and cod-liver oil by Malcolm [1926] who pronounced it to be "one of the richest known natural sources of vitamin A."

Rosenheim and Webster [1927], however, point out that this statement requires modification, since in his animal tests Malcolm did not give a constant supply of vitamin D. The above-named workers were unable to obtain a supply of mutton bird oil. They therefore examined the stomach oil of the fulmar petrel (*Fulmarus glacialis*) which breeds in enormous numbers in St Kilda, and which corresponds most closely to the antarctic mutton bird. They found that when a constant supply of vitamin D was given throughout the experiment, the vitamin A content was no better than that of a good Newfoundland cod-liver oil.

These workers also demonstrated the presence of vitamin D in the stomach oil of the fulmar petrel, and found that "a dose of 20 mg. gave partial protection, whilst 40 mg. completely protected the animals against rickets."

By the kindness of the African Industries Trade Service, some mutton bird oil was obtained for the following experiments.

Two litters of rats were used. No. 1 was a litter of six white rats weighing 39–50 g., and No. 2 a litter of twelve black-and-white rats, weighing 36–46 g. at the beginning of the experiment. The basal diet given was McCollum's No. 3143 low phosphorus, rickets-producing diet. The doses of mutton bird oil were given daily from the start of the experiment. A series of control animals receiving various doses of a good cod-liver oil was included in order to provide a positive control for the experiment. As will be seen from the table, the doses were given in multiples of five, starting from a daily dose of 0·2 mg. of the petrel oil and culminating in a dose of 120 mg.

Litter No. 2 was killed at the end of 23 days and litter No. 1 at the end of 24 days. To test the condition of the skeletons of the rats, the junctions of the 6th and 7th ribs on the right side were examined histologically, and chemical analyses were made of the leg-bones (femora, tibiae and fibulae). The methods used are described in full in the paper by Chick, Korenchevsky and Roscoe [1926].

In comparing the effect of the two oils, both the chemical and histological analyses indicate that petrel stomach oil has a vitamin D value equal to about 1/5 of that of the cod-liver oil. Doses of the former of 5, 25 and 120 mg. afforded respectively about the same degree of protection as 1, 5 and 25 mg. of the latter. For the rats receiving the above three doses of petrel oil the average value of the A/R ratio (ratio of the mineral ash to the organic matter in the fat-extracted bones) was 0·64, 0·74 and 0·92 respectively; for rats receiving the above-stated cod-liver oil doses, it was 0·59, 0·81 and 0·93. Similarly, the mean values of the percentage of ash contained in the fat-extracted dry bone were 39·1 and 43·8 for animals receiving respectively daily doses of 5 and 25 mg. petrel oil and 39·6 and 44·6 for those receiving cod-liver oil in doses of 1 and 5 mg. respectively (see table).

Rat	Litter	Sex	Oil	Dose (mg.)	A/R	Average	% ash in fat-extracted dry bones		Degree of rickets, from histology of rib junctions
31	1	♀	Petrel	0·2	0·474	0·50	31·75	35·2	Severe rickets
37	2	♀	,,	0·2	0·632		38·74		Rickets
33	1	♀	,,	1·0	0·461	0·45	31·55	35·4	Severe rickets
39	2	♀	,,	1·0	0·443		35·77		,, ,,
40	2	♂	,,	1·0	0·448		38·94		Rickets
34	1	♀	,,	5·0	0·639	0·64	38·99	39·1	Severe rickets
43	2	♀	,,	5·0	0·647		39·28		,, ,,
35	1	♂	,,	25·0	0·548	0·74	40·84	43·8	Severe rickets
44	2	♀	,,	25·0	0·801		44·48		Slight rickets and osteoporosis
47	2	♂	,,	25·0	0·858		46·17		Osteoporosis only
48	2	♀	,,	120·0	0·920	0·92	47·91	47·9	Nearly normal; some osteoporosis
32	1	♀	Cod	1·0	0·535	0·59	34·84	39·6	Severe rickets
38	2	♀	,,	1·0	0·750		42·86		Slight rickets
41	2	♂	,,	1·0	0·486		41·18		,, ,,
42	2	♀	,,	5·0	0·806	0·81	44·62	44·6	Slight rickets and osteoporosis
36	1	♂	,,	25·0	0·962	0·93	49·03	46·2	Osteoporosis only
45	2	♀	,,	25·0	0·932		48·23		,, ,,
46	2	♂	,,	25·0	0·904		47·47		,, ,,

The histology of the rib junctions of animals receiving 5 mg. of petrel oil or 1 mg. of cod-liver oil showed decided rickets. This condition was much modified when the daily doses of the oils were increased to 25 mg. of petrel and 5 mg. of cod-liver oil respectively, and where the daily ration was further increased to 120 mg. of petrel or 25 mg. of cod-liver oil, osteoporosis only was seen.

Summary and conclusions.

The presence of vitamin D in amount equal to about 1/5 of that present in a good cod-liver oil, has been demonstrated in the stomach oil of the Australasian mutton bird (*Aestralata lessoni*).

How the vitamin D came into the stomach oil is unknown. This oil appears to consist mostly of cetyl esters and therefore not to be the undigested fatty residue from the "whale-feed." However, the fact that the mutton bird feeds on the same crustacean ("whale-feed") as the Australian red cod, seems to suggest that this might be the common source from which both bird and fish draw their supplies of vitamin D.

My thanks are due to Prof. C. J. Martin, Dr Chick and Prof. Korenchevsky, for their kind help and criticism, to the African Industries Trade Service for the sample of petrel oil used in the experiments, to the Medical Research Council for a whole time grant and to the Lister Institute for the hospitality of its laboratories.

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XCVII. THE SYNTHESIS OF THE ANTINEURITIC FACTOR (TORULIN) BY YEAST.

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(*Received May 4th, 1927.*)

There has been some uncertainty as to whether yeast could synthesise vitamin B when grown on a medium which did not contain it. Macdonald [1922], Heller [1923], and Nelson, Fullmer and Cessna [1921] tested for the factor promoting growth in rats and found it present. Harden and Zilva [1921] and Heller [1923] tested for the antineuritic factor, and found this to be synthesised. But Eijkman, Hoogenhuijze and Derks [1922] found that the antineuritic factor did not occur in yeast unless the medium contained either it or its decomposition products.

In all these experiments there seems to have been no test at the end as to the success of the precautions against bacterial contamination, although it is known that some species of bacteria can synthesise vitamin B [Damon, 1923; Heller, Elroy and Garbeck, 1925]. In some of the experiments it is almost certain that unsuspected impurities containing bios were present [Hoet, le Clef and Delrue, 1924; Funk and Freedman, 1923; Willaman and Olsen, 1923]. Hence further experiments were undertaken to investigate whether yeast could synthesise the antineuritic factor, if the possibility of active impurities in the medium and of bacterial contamination was excluded or controlled. The antineuritic factor is considered here to be the factor curative for symptoms of head retraction in pigeons fed upon polished rice.

Method.

The medium used was:

KH_2PO_4	5 g.	Cane sugar ...	50 g.
NH_4Cl	2·5 g.	Water	1 litre
$MgSO_4$	0·35 g.	Bios extract ...	3 cc.
$CaCl_2$	0·25 g.		

The cane sugar was recrystallised three times from 80 % alcohol. The salts were either purified by double recrystallisation, or Kahlbaum's preparations were used. All glassware was cleaned with chromic acid. The water used was glass-distilled from alkaline permanganate.

The bios extract deserves further explanation. It was prepared by Mr G. L. Peskett from baker's yeast, according to the method of Eddy, Kerr, and Williams [1924], up to the "fuller's earth" stage, and its activity may be

judged from the following experiment (Table I). Flasks containing 100 cc. of the salt solution and various concentrations of the extract were inoculated, and after growing for 6 days at 33° the yeast was centrifuged off, dried at 140° overnight, and weighed.

Table I.

Conc. of bios extract %	Dry wt. of culture g.	Wt. of yeast / Conc. of bios
1·0	0·10, 0·15 = 0·125	0·125
0·5	0·10, 0·14 = 0·12	0·240
0·2	0·07, 0·10 = 0·085	0·425
0·1	0·04, 0·04 = 0·04	0·400
0·025	hardly any growth	—

The extract also contained a certain amount of vitamin. The attempt was made to control this by using low concentrations of extract in the medium. The content of vitamin may be obtained from the following results (Table II), obtained by the curative and protection test [Kinnersley and Peters, 1925].

Table II.

Pigeon	Dose of extract cc.	Duration of cure
1	1	No cure
2 (re-treated bird)	5	2 days
3	5	7 „
4	4·5	6 „
5	4·5	1 day

Prof. Peters makes the following comment upon the figures.

"The bios extracts are distasteful to the birds and the results obtained exceptionally variable. This is apt to be so with inactive concentrates, and it is usually found that re-treated birds react less readily to curative extracts than birds fresh from the dealers. For the purpose of this paper it would appear wisest to take the least favourable results, namely, experiments 3 and 4."

Hence, for the purpose of this paper, it may be concluded that the extract contained not more than 1·4 day doses per cc., *i.e.* 2·1 day doses per 500 cc. medium as a maximum.

The strain of yeast used was *Saccharomyces cerevisiae* Nat. Type Culture No. 381/21, from the Lister Institute. One drop of a 48 hours' growth in the above medium was transferred to 10 cc. of the salt solution, and one drop of this was used to inoculate each of the 500 cc. culture flasks. The inoculation contained about 20,000 cells. The flasks were incubated for 5 days at 30–33° and then the yeast was centrifuged off and extracted twice with 70 % alcohol as described by Funk, Harrow and Paton [1923].

Before centrifuging, smears were made of the yeast grown and subcultures of the supernatant fluid were also made on to agar slopes. These were incubated for 2 days at 33° and one at 37°, and then a composite smear was made from

each tube. The two sets of slides thus obtained were examined for bacterial contamination, staining with carbol-thionine blue. Careful inspection showed it slight, but an attempt was made to give some objective indication of the degree to which it was present. Three microscope fields on each slide were taken at random, and everything that might possibly be a micro-organism was counted, the result being expressed as a percentage of the number of yeast cells present in the same field (300–500). When expressed thus, the contamination of the actual yeast extracted was about 1–2 % (see below). This is an outside figure and is probably excessive, especially as the agar slopes —presenting conditions favourable to bacterial growth—gave a figure only slightly higher (2–3 %). In any case, the proportion by volume was very much less, since a yeast cell is at least 250 times as large as an average micro-organism.

Results.

A series of earlier experiments was spoilt by the presence of too much bios extract, and so, of too much vitamin in the culture medium. The latest results obtained with the most improved technique were as follows:

Table III.

Pigeon	Yeast wt. g.	Cured or protected for days	Contamination	
			Smear %	Agar slope %
5	3·7	3	1·3	—
6	3·9	5	1·3	3·0
7	3·85	3+	1·5	2·2
8	2·95	7	2·1	—

Pigeon 8 came from an irregular batch of which several other birds gave abnormally high results, and must therefore be discarded. For the purpose of the experiment, pigeons 5 and 7 gave the lowest result, namely, 3 days. If this lowest figure is therefore taken for the curative effect of the yeast, and compared with the highest figure obtainable for the amount of possible vitamin in the bios present in the medium, the results obtained will be the most unfavourable to synthesis found under the conditions of the experiments. The facts are that not more than 2·1 day doses/500 cc. were originally present, whereas not less than 3 day doses were found in the yeast after growth. A balance of synthesis of 1 day dose out of three is therefore indicated.

Discussion.

The increase of activity observed is small, and it might be suggested that it could be attributed to unrecognised impurities present in the medium. Though the salts and sugar had been purified as described, an attempt was made to extract the medium with successive fractionations with 70 % alcohol. This had to be abandoned owing to the difficulty of freeing from sugar sufficiently to give to a bird. Against the possibility of slight contamination in the medium, however, may be set the probability that Funk's method does

not extract all the torulin present. Further, no allowance can be made for a possible utilisation of the factor by the yeast in the course of its metabolism. The figures given for the bacterial contamination which are maximal suggest that this possible source may be neglected.

A general consideration of the problem of synthesis by the yeast cell suggests that such synthesis is likely to be small unless a much purer bios preparation can be obtained. It is inherently probable that an optimal concentration of the vitamin in the yeast cell can be obtained either by intracellular synthesis or adsorption from the environment. Fluid in which yeast has grown is notably free from vitamin, a fact which was confirmed in some of the earlier experiments. Two lots of 300 cc. of medium, after growth of the yeast, were extracted with 66 % alcohol and taken up in aqueous solution but failed to cure birds, although initially 4 day doses were present in each. Adsorption of vitamin by the yeast has been stressed by Eijkman *et al.* [1922], Randoin and Lecoq [1926] and Southgate [1924]. It may be concluded that yeast preferentially adsorbs the factor, merely synthesising when necessary to meet the optimal requirements. Therefore until a bios preparation can be obtained containing a growth-promoting factor of the bios type in much larger concentration than it does torulin or its decomposition products, the problem would seem impossible of final settlement. There were two other contentions, contained in Eijkman's paper. It was suggested that yeast could not synthesise the antineuritic factor, but merely reactivated its decomposition products. In these experiments, treatment with alkali was avoided, and the method of preparation of the bios should not be destructive of torulin. It is, however, still possible that the apparent synthesis is merely a reactivation of some decomposition product. The further contention that, as the antineuritic factor is not synthesised by yeast, it is to be distinguished from the growth-promoting factor, is puzzling to interpret in the light of recent work upon the duality of vitamin B. If both factors are needed for the growth of the rat, some synthesis of the antineuritic vitamin must have occurred.

SUMMARY.

Evidence is produced to show that yeast (*S. cerevisiae*) can synthesise the curative factor for pigeons (torulin) in small amounts.

I wish to acknowledge my indebtedness to Mr G. L. Peskett for his invaluable advice in connection with the growth of yeast cultures, and to Mr W. H. Kinnersley for testing out the extracts on the pigeons.

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XCVIII. ON OVOMUCOID.

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I. The carbohydrate content of the ovomucoid molecule during the development of the chick.

When the carbohydrate metabolism of the extra-embryonic part of the developing hen's egg was being considered in a previous paper [Needham, 1926], an attempt was made to gain some idea of the movements of the "ovomucoid" fraction by adding the glycogen-glucose to the free glucose and subtracting the value so obtained from the total glucose. The resulting curve (being admittedly only of exploratory significance) gave a peak in the middle of incubation between its initially high values and the minimal ones of the end of incubation. This conflicted with the results of Bywaters [1913], who by means of analyses of coagulable and non-coagulable nitrogen in the egg-white during incubation concluded that there was no preferential absorption of albumin or ovomucoid but that both diminished steadily in the egg-white as development proceeded. Assuming that Bywaters' technique was satisfactory, as may most probably be done, there could be only two explanations of this discrepancy, (*a*) that the ovomucoid molecule contained more glucose in its prosthetic group at some times than at others during incubation, or (*b*) that the hump in the ovomucoid curve was due to a temporary increase of mucoprotein in the yolk only, at the time in question, mucoprotein which might or might not possess the same amount of glucose within its molecule as the ovomucoid of the egg-white. The former possibility was made somewhat likely by the recent work of Komori [1926], who considers that the glucose of ovomucoid is united to the protein part in the form of a polysaccharide, but if it were true, the results of Bywaters who found the ratio

$$\frac{\text{g. uncoag. N per 100 g. egg-white}}{\text{g. total glucose per 100 g. egg-white}}$$

to be more or less constant during incubation, would have to be abandoned.

In order to settle this question, therefore, preparations were made of ovomucoid from the egg-white from the 5th to the 18th day of incubation. The method used was as follows. The eggs were candled and pencil marks made on the shells to indicate the clear part where the egg-white lay close to the shell-membrane. They were then opened in that region, and the egg-white was carefully collected, though no attempt was made to recover every trace. The chalazae were included with the white. The mass was then weighed in a

tared bottle, diluted to twice its volume with distilled water and placed on a shaking machine for 2 hours in order to break up the albumin. For this purpose a quantity of broken porcelain was added and a few drops of caprylic alcohol to prevent frothing. The mixture was then further diluted and heated in a boiling water-bath for an hour with acetic acid, the reaction being so adjusted as to remain green to bromocresol purple. In this way the albumin was almost completely removed, subsequent tests with trichloroacetic acid demonstrating the presence of only the slightest traces of albumin. The filtrate was next poured into ten times its volume of 97 % alcohol, and the flocculent white precipitate filtered off, dissolved in as small an amount of hot water as possible, and reprecipitated. It was finally dried well over calcium chloride in a vacuum desiccator, and eventually hydrolysed for 4 hours with 5 % hydrochloric acid, the glucose or glucosamine set free being estimated in the filtrate by the Hagedorn-Jensen method [1923] after precipitation with phosphotungstic acid, which does not remove glucosamine.

Table I. *Experiments on ovomucoid.*

1	2	3	4	5	6	7	8	9	10	11	12
Day	No. of egg-whites	Total wt. g.	Egg-white g. per egg	Col. 4 smoothed	Wt. of egg-white in % of whole egg	Pure ovomucoid isolated mg.	Col. 7 mg. per egg	Col. 8 smoothed	Ovomucoid in mg. % of wet wt. of egg-white	Mg. taken for estimation	% glucose
0	—	—	—	—	—	—	—	—	—	—	—
1	—	—	—	—	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—	—	—	—
3	—	—	—	—	—	—	—	—	—	—	—
4	—	—	—	—	—	—	—	—	—	—	—
5	32	387	12·1	11·8	21·15	3583	112	128	1080	453	11·40
6	31	340	10·9	11·6	21·02	774*	25	125	1080	254	8·44
7	36	418	11·7	11·5	20·95	380*	11	122	1060	380	7·38
8	25	278	11·1	11·3	20·77	3563	143	119	1053	643	13·35
9	19	218	11·5	11·0	20·40	1015	53	117	1065	345	7·99
10	17	180	10·6	10·6	19·74	1427	84	110	1039	447	6·40
11	14	150	10·1	10·0	18·80	960	69	103	1030	310	12·80
12	18	173	9·6	9·3	17·60	2405	134	92	989	785	9·39
13	9	73	8·2	8·3	15·85	584	65	82	988	414	12·90
14	29	335	7·6	7·1	13·68	522	18	70	986	126 176	16·10 13·00
15	3	13	4·3	5·5	10·70	286	95	55	1000	185	11·30
16	44	248	3·02	3·9	7·65	2562	58	40	1026	360 652	14·30 13·00
17	70	337	3·1	2·2	4·36	3166	45	20	909	380 516	14·70 12·60
18	11	12	1·1	0·6	1·20	49	4	4	—	—	—
19	—	—	—	—	—	—	—	—	—	—	—
20	—	—	—	—	—	—	—	—	—	—	—

* Considerable losses.

Table I shows the results in general. These are the first figures to be published for the weight of the egg-white at different stages, and it is interesting to note how it disappears altogether by the 18th day. Quantitative expression is thus afforded for what originally was an observation of William Harvey [1653]. Col. 6 shows what percentage of the whole egg is taken up by the white, and is calculated, allowing for the loss of water during incubation, from

the figures of Murray [1925, Fig. 2]. It descends from 21 to 0 %. Col. 7 shows the ovomucoid isolated in g. and cols. 8 and 9 in g. per egg; it falls from 0·128 to 0·004. Although it is not claimed that there was a quantitative recovery of ovomucoid (and indeed these figures are much less than the fragmentary ones of Komori) yet, as the same care was used all through, it is probable that they have some relative significance. When they are compared with those of col. 5 and expressed as percentage of the wet weight of egg-white, as is done in col. 10, it is interesting to note that the amount of ovomucoid in 100 g. of wet egg-white remains constant throughout development at about 1 g. As far as these figures go, then, they confirm the observations of Bywaters and indicate that there is no preferential absorption of ovomucoid from the white, although there may be from the yolk. This latter possibility is made likely by the fact that the hump on the ovomucoid-glucose curve comes just between the two times at which the mucoprotein-glucose inside the embryo is high.

Col. 11 shows the mg. of ovomucoid taken each day for hydrolysis, and col. 12 the values found for the carbohydrate content of the molecule calculated as glucose. The average value is 11·5 %. Probably this represents the most accurate figure so far obtained, for all previous estimations were performed with copper methods and, as Holden [1926] has shown, in no conditions are these more unreliable than when dealing with protein hydrolysates. The values are tabulated as follows:

Investigator	% glucose in ovomucoid
Hofmeister [1898]	15·0
Seemann [1898]	29·4
Langstein [1900]	10·5
Willanen [1906]	19·5 to 22·3
Pavy [1907]	21·7
Samuely [1911]	34·9
Neuberg and Schewket [1912]	24·0
Zeller [1913]	33·7
Average	23·4
Present results	11·5

The most striking thing about col. 12, however, is that when the figures are plotted on a graph, an unmistakable upward trend is seen. The line which is drawn, having regard to all the points, traverses the graph, rising all the time from 7·4 on the 5th day to 15·4 on the 18th. No explanation has been found for this phenomenon. It is at any rate clear that there is no marked increase in the glucose content of ovomucoid at the period when it would be expected in the former of the two views outlined above. The mucoprotein of the yolk is therefore probably much increased at that time, and this will henceforth be used as a working hypothesis.

The gradual trend of the ovomucoid toward a higher glucose content may be regarded as one of those slow intramolecular changes which take place during embryonic development, a good example of which has recently been afforded by Plimmer and Lowndes [1927] in their work on the amino-acid distribution in the proteins of the hen's egg before and after incubation.

II. The existence and localisation of ovomucoidase in the hen's egg.

In 1899 Müller and Masayama [1899, 1900] reported the discovery of an active enzyme in the yolk of unincubated hens' eggs which would hydrolyse starch. This diastase was destroyed by heat and "under favourable conditions" would convert 45 % of a 3 % starch solution into the soluble forms of dextrin and isomaltose in 24 hours at 37°. This put on a sure basis the earlier and rather doubtful results of Krukenberg [1879] and was in turn confirmed by Diamare [1909], Herlitzka [1906] and Roger [1908]. Idzumi [1924] brought forward data which showed that the activity of the extra-embryonic diastase markedly increased as development proceeded, especially after the 15th day (rising from 40 to 640 units). Finally, Komori [1926] demonstrated that most if not all of the glucose in the ovomucoid molecule could be liberated by incubation with diastase prepared from meal. It remained to demonstrate that the egg itself, or certain parts of it, possessed the power of hydrolysing ovomucoid.

Experiments with this aim in view were carried out as follows. About fifty eggs were incubated under standard conditions for 5 days. At the end of that time they were broken open and divided into fractions thus: (*a*) the embryonic body, (*b*) the blastoderm and yolk-sac, (*c*) the egg-white, and (*d*) the yolk. The embryos themselves were placed one by one in distilled water in a small crystallising vessel under a strong beam of light and carefully freed from their amniotic membrane and the small allantois. After having been well washed in distilled water they were ground up with clean quartz sand in a mortar. The blastoderms and yolk-sacs were collected by pouring yolks and whites together into a large volume of water from which the membranes could with care be fished out. They were washed for 6 hours in a strong stream of water and finally ground up with quartz sand. The yolk and white were collected from other eggs with as little contamination as possible and were placed on a shaking machine for 2 hours with broken porcelain in order to mix them well. Then into a series of conical flasks were pipetted 50 cc. each of a solution of pure 5th day ovomucoid previously prepared and so arranged that each trial should contain 400 mg. of ovomucoid. Into a parallel series of flasks were run 50 cc. each of water, and to one of each series were added 50 cc. of embryo extract, yolk, etc., as the case might be. Toluene was added to 10 %. The flasks were plugged with cotton wool, and incubated at 37° for 3 days exactly, being shaken once a day. In view of the preliminary nature of these experiments, the p_H was not controlled by buffers. Glucose liberated was estimated in all the samples by the Hagedorn-Jensen method following precipitation with phosphotungstic acid. Table II gives the results.

As can be seen from Exp. K the ovomucoid preparation itself contained no free glucose at all, but when incubated alone about 2 mg. were split off, perhaps because of the presence of minimal amounts of the enzyme. This auto-digestion effect must be subtracted from the apparent results of col. 6 and the corrected values are shown in col. 7. It will be noted in the first place that the

Table II. *Experiments on ovomucoidase.*

1	2	3	4	5	6	7	8	9	10	11
Exp.	Fraction tested	Substrate added	3 days' incubation at 37°	mg. glucose found	Difference due to ovomucoid hydrolysis	Corrected for Exp. L	Yield % of theoretical	Dry wt. of sample g.	Col. 7 as % dry wt.	p_H of system during incubation
A	Embryo extract	−	+	5·83	0·49	0·00	0·00	0·351	0	7·0
B		+	+	6·32						
C	White	−	+	5·04	2·31	0·44	1·49	6·00	7·3	8·1
D		+	+	7·35						
E	Yolk	−	+	17·70	11·10	9·23	31·20	15·00	61·6	5·4
F		+	+	28·80						
G	Yolk-sac and blastoderm	−	+	0·15	3·98	2·11	7·13	—	—	6·9
H		+	+	4·13						
K	Water	+	−	0·00	—	—	—	—	—	7·1
L		+	+	1·87						

slight excess of glucose in the embryo preparations is much more than accounted for by the correction of Exp. L, the effect of the egg-white is much reduced, but the yolk and the yolk-sac results still retain considerable magnitude. The conclusion, therefore, is that the enzyme ovomucoidase is not contained in the embryonic tissues themselves and only to a negligible extent in the egg-white, but that the yolk is very rich in it and that the blastoderm and yolk-sac also show a fair activity. Thus the embryo tests yield 0 % of the theoretical, the white 1·49, the yolk 31·2 and the yolk-sac 7·13. This conclusion is not affected by the varying amounts of solid material which must have been contained in the original samples, for as cols. 9 and 10 show, the yolk even so retains its superiority.

These results fit in well with what is already known. Roger, for instance, found more diastatic activity in the yolk than in the white. Bywaters [1913], by comparing the ratio

$$\frac{\text{g. uncoag. N per 100 g. egg-white}}{\text{g. glucose per 100 g. egg-white}},$$

came to the conclusion that the ovomucoid of the egg-white was not split up into protein and sugar moieties before absorption; but he only investigated the white, and for that fraction alone his results do not conflict with those now brought forward. The ovomucoid must be pictured passing from the white to the yolk, and there being split up into free glucose and protein before absorption into the embryo through the vitelline veins. It is interesting that the embryo possesses no ovomucoidase or very little; the hydrolysis must therefore be considered to take place outside it. The passage of ovomucoid from white to yolk is not difficult to imagine, for the existence of a current of water and of free glucose yolkwards has been clearly demonstrated [Aggazzotti, 1919; Needham, 1927]. Idzumi's observations on the increase of activity of the yolk diastase during development fit in perfectly with the increasingly rapid

disappearance of ovomucoid seen in Table I, col. 9. It would be very desirable to extend these observations by investigating the effect of p_H, salt concentration, etc., on the enzyme and by determining whether its activity remains the same in all fractions during development.

SUMMARY.

1. The carbohydrate content of the ovomucoid molecule, calculated as glucose, appears to be on an average 11·5 %. But there is a definite trend upwards as development proceeds, only 7·4 % being present on the fifth day but as much as 15·4 % on the 18th day.

2. An enzyme which hydrolyses ovomucoid appears to exist in the yolk and the yolk-sac of the hen's egg at the 5th day of development, possibly also in the white and the blastoderm, but not in the embryo.

3. Utilisation of ovomucoid by the embryo probably consists in a regular passage of ovomucoid into the yolk, where it is hydrolysed before being absorbed through the vitelline veins at a rate which varies during the incubation period.

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XCIX. A QUANTITATIVE STUDY OF SUCCINIC ACID IN MUSCLE. II.

THE METABOLIC RELATIONSHIPS OF SUCCINIC, MALIC AND FUMARIC ACIDS.

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(*Received April 30th, 1927.*)

Introduction.

That the succinic acid content of fresh, chopped muscle tissue increases on incubation in buffer solutions of suitable p_H has been shown in a previous paper [Moyle, 1924]. It would obviously be of the greatest assistance towards elucidating the rôle of the succinic acid in the intact muscle if one could discover whether the succinic acid maximum reached *in vitro* is an equilibrium point or not; that is to say, whether the formation of more succinic acid is hindered by the accumulation of the acid. If this were the case, we might expect that, after removal of the accumulated acid by admission of oxygen, putting the system back under anaerobic conditions would lead to a renewal of the maximum from some unknown precursor.

When such experiments were performed, it was indeed found that after a period in nitrogen, followed by a period in oxygen involving decrease in succinic acid content, a return to nitrogen was accompanied by rise in concentration of this acid.

There is of course an alternative explanation to the one just put forward of the renewed succinic acid maxima: that the fumaric and malic acids formed in presence of oxygen [Batelli and Stern, 1911, 1; Einbeck, 1919] are themselves not oxidised or only very slowly; when anaerobic conditions again prevail these acids might be reconverted into succinic acid. As regards the possibility of the removal of fumaric and malic acids by oxidation, Batelli and Stern [1911, 2] showed that these acids, as well as citric acid, were oxidised to some extent by chopped muscle suspended in three times its weight of water, though the oxygen uptake is very much less rapid than in the oxidation of succinic to fumaric acid. Thorough washing they found to destroy the power of the tissues to bring about the former oxidations. Other workers [Thunberg, 1909, 1911, 1 and 2; Meyerhof, 1919; Grönvall, 1924] have observed increased oxygen uptake upon adding fumaric acid to muscle after various degrees of extraction. The oxidation of all these acids is inhibited by the presence of

inorganic salts [Batelli and Stern, 1911, 2], but with the fumaric and malic acids, if we may judge from the figures given for citric acid, not more than with the succinic acid.

When the alternate rise and fall in succinic acid content had been established, it therefore became necessary to determine the amounts of fumaric and malic acids present at the same times, and a method is described for estimating the malic acid, as well as the total quantity of the three acids together, in the same muscle sample. From these two values, as is explained later, a rough calculation may be made of the succinic and fumaric acids present. Using these methods, the experiments with alternate atmospheres were repeated, and it was found that the total content of the three acids does not remain constant, but rises and falls markedly with the succinic acid content. Simple interconversion of succinic acid on the one hand and the oxidation products malic and fumaric acids on the other, is not then the explanation of the results obtained.

The effect of alternate aerobic and anaerobic conditions on the succinic acid content.

The first result which emerged from the following experiments was the difficulty of obtaining complete disappearance of the succinic acid in oxygen, although, as is well known, succinic acid added to washed muscle is rapidly and quantitatively converted into fumaric acid [Fleisch, 1924].

Muscle removed from the neck of the bullock immediately after slaughtering was brought to the laboratory in a pan surrounded by a freezing mixture; a long, cylindrical muscle could be dissected out with little injury, and this, after further cooling, was minced and samples were weighed out. After the required time of incubation the flask was cooled, and an equal volume of alcohol was added (counting each g. of muscle as 1 cc. in the suspension). The succinic acid was estimated according to the method previously described.

Exp. 1. Four 50 g. lots were used, each suspended in 100 cc. of buffer solution at p_H 7·4. Two of the flasks also contained about 50 mg. each of succinic acid. One of these flasks and one control were connected to a nitrogen cylinder, the remaining two to an oxygen cylinder. All were left at 33° for 1·5 hours.

	Succinic acid in mg. per 50 g.
Control in oxygen	8·5
,, +succinic acid in oxygen...	28·8
Control in nitrogen	12·8
,, +succinic acid in nitrogen	54·4

It seemed possible that the conditions might be unfavourable for complete oxidation, either of the succinic acid to fumaric, or of the fumaric acid further (with consequent retardation of fumaric acid formation) owing to the inhibitory effect of the phosphate in the buffer and of too low a p_H (according to Ohlsson the optimum p_H is about 8·7). A buffer solution much less concen-

trated was therefore made up, following the directions given by Ohlsson [1921] for the buffer that he used with his enzyme preparation. The p_H of this solution was 9·0, and it was ascertained by means of the hydrogen electrode that the addition of 40 g. of beef to 500 cc. did not cause the value to fall below 7·47, even after several hours.

Exp. 2. 40 g. lots of beef were used, each suspended in 500 cc. of the dilute buffer; 50 mg. of succinic acid were added to each bottle and both were oxygenated at 37°.

	Succinic acid in mg. per 40 g.
After 40 mins.	28·2
After 60 mins.	28·8

It is clear that these precautions have not overcome the difficulty of incomplete removal, which must be due to some other factor than the effects of the medium already considered. For instance, it is possible that under the conditions of the experiment continuous production of succinic acid is taking place, while with washed muscle the precursor has been removed. Further attempts, therefore, to obtain complete disappearance were abandoned, and the experiments on anaerobiosis and oxygenation were begun.

In the remaining experiments described in this paper a somewhat different arrangement was used; the desired atmosphere was obtained by evacuating the flasks till the contents boiled, and then filling with the required gas until a little more than atmospheric pressure was reached. As it had been found that pigeon breast muscle gave a higher yield of succinic acid than any other kind tried, this muscle was used; four pigeons were generally taken for each experiment. After killing, bleeding and plucking, they were placed breast downwards on ice for a short time; then the two breast muscles were dissected off from each with as little injury as possible, further cooled in a covered glass dish packed in freezing mixture, and minced. As the succinic acid content varies considerably from one pigeon to another, very thorough mixing was essential before samples were weighed out. The anaerobic flasks were always evacuated before removal from the bath, and were opened with a side-tube dipping under alcohol, so that the contents were precipitated without admission of air. The oxygen flasks were shaken almost continuously.

Exp. 3. Temp. 33°. Phosphate buffer, p_H 9·0 (1·4 % Na_2HPO_4, $2H_2O$); two volumes used.

1.0 p.m.	All flasks filled with nitrogen.
2.0 „	First flask, N 1, removed; oxygen introduced into remaining four.
2.40 „	Second flask, O 1, removed; nitrogen introduced into the others.
3.20 „	Third flask, N 2, removed; oxygen introduced into remaining two flasks.
4.0 „	Fourth flask, O 2, removed; nitrogen introduced into the last flask.
5.15 „	Last flask, N 3, removed.

The results are shown in Fig. 1.

Exp. 4. Temp. 33°. The concentration of phosphate was halved and the volume used was doubled. All the periods were shortened (see Fig. 1). The results were very like those in Exp. 3.

Exp. 5. This experiment was similar to the last, except that at the end of

the first nitrogen period all the flasks but N 1 were opened and 20 cc. of 0·2 *N* sodium hydroxide were added to each. It was calculated that this should raise the p_H by about one unit, and the final p_H was found to be about 7·3 instead of below 7·0, as in the previous experiment. This change in the treatment does not seem to affect the results (Fig. 1).

The combined results of these three experiments seem to show that, as time goes on, the possibility of the complete removal of succinic acid in oxygen becomes progressively greater. When the second oxygen period falls, 3 hours after the beginning of the experiment, disappearance is almost complete. Renewed maxima in nitrogen are obtained, but the value in each is generally somewhat lower than in the previous one.

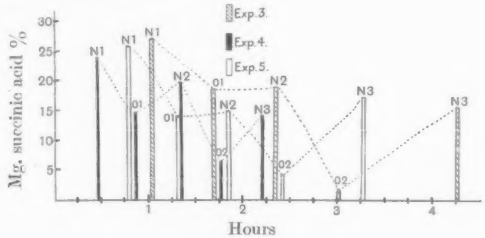

Fig. 1.

THE METHOD OF ESTIMATION OF MALIC ACID AND OF THE THREE ACIDS TOGETHER.

In the method used for the estimation of the total quantity of the three acids, they were quantitatively precipitated as the silver salts by adding $AgNO_3$ to a neutral solution in 36 % alcohol; the silver was then determined by titration with *N*/100 KCNS. After removal of the silver precipitate, the malic acid was estimated polarimetrically as the molybdenum compound, which, as is well known, shows an enormously increased rotation.

As two important difficulties were encountered in the working out of the method, it might be well to state the reason for some of the processes described.

(1) It was desirable to extract with ether before the silver precipitation to get rid of traces of amino-acids, purines, etc., and also the greater part of the phosphate (if the whole of the phosphate of the buffer is removed with barium, there is much loss by adsorption of the acids, especially succinic, on the barium phosphate precipitate). On account of the high partition coefficient of malic acid between water and ether (62 : 1) it was necessary to put the solution on to some water absorbent, and to extract this in a Soxhlet. As water absorbents, plaster of Paris, anhydrous sodium sulphate and fat-free filter paper strips were tried, but found useless because the malic acid, once adsorbed, could not be quantitatively removed. Only about 66 %, and sometimes much less, could be recovered when the pure acid was tested. In the presence of high concentrations of salts, *e.g.* ammonium sulphate, in the liquid adsorbed, the

recovery was much less; and when a muscle extract with added malic acid was tried, none of the acid could be recovered. Using silica gel[1], on the other hand, 90 % of the acid could be found again after 6 hours' extraction, when the pure acid was tried; and 84 % was recovered after 24 hours' extraction in the presence of phosphate and lactate in amounts comparable to those in a muscle sample. No explanation of these phenomena inhibitory to extraction can be offered; in the case of the lactate some indications were obtained that, even in the presence of silica gel, the malic acid only begins to be extracted when all or most of the lactic acid has been extracted, and for this reason the time of extraction was made lengthy.

(2) In the presence of so large a proportion of lactic acid, it was found that the usual precipitation methods for malic and succinic acids, especially the former, fail. Thus for succinic acid alone very good results were obtained for 10 mg. of the acid by precipitating the calcium salt in 87 % alcohol, and, after filtering and slight washing, dissolving the precipitate in water and estimating as the silver salt; but on addition of 150 mg. lactic acid, practically no precipitate was obtained on adding alcohol. Using the barium salts, in presence of 300 mg. lactic acid, 10 mg. succinic acid was precipitated to the extent of 70 % only. 300 mg. lactic acid almost entirely prevents the precipitation of 10 mg. malic acid whether as the calcium or the barium salt in 85 % alcohol.

Similar results were obtained for the silver methods. Precipitation in 36 % alcohol solution (3 cc. alcohol to 5 cc. water) had been adopted because, in this medium, at p_H 6·2–7·2 the three acids are quantitatively precipitated as the silver salts, whilst in aqueous solution only about 60 % of the malic acid is precipitated. When lactic acid was present, however, to the extent of 40 times the malic acid, only about 50 % of the latter was precipitated; the succinic acid precipitation was less affected. In order to overcome this difficulty, the practice was adopted of a preliminary silver precipitation in 75 % alcohol; in this way practically all the lactic acid is removed, and the precipitate obtained, which gives a high percentage of silver and probably contains basic salts, is dissolved and re-precipitated as described later. This double precipitation is unnecessary if the proportion of lactic to malic acid is not greater than 20 : 1, but, as we shall see, it is usually greater than this in muscle.

Complete removal of the lactic acid is also necessary for the polarimetric estimation of malic acid, as the rotation of the former acid is also markedly increased on addition of molybdenum.

The details of the method finally used were as follows. The 50 % alcoholic extract is strained from the muscle residue by pressing through muslin, and the muscle washed twice with 50 % alcohol. The extract is filtered through paper, neutralised, and evaporated *in vacuo* at 40°. When the volume is reduced to about 75 cc., the precipitated protein is filtered off and the filtrate

[1] The commercial name of the product used was "Super-Cel Hyflo," kindly supplied to me by the makers, the Celite Products Corporation.

evaporated on the water-bath to about 7 cc.; it is cooled on ice and 2 cc. of 50 % H_2SO_4 (by volume) added. The acid extract is now adsorbed on a silica gel, the dry powder is well mixed and ground, and placed in a Soxhlet thimble. After 1 hour's extraction with light petroleum to remove most of the fat, it is extracted for about 90 hours with ether.

When the extraction is finished, a little water is added, and the ether is distilled off. The warm aqueous solution is neutralised with saturated baryta solution to remove sulphate and phosphate. The neutralised solution should not be allowed to stand very long or remain very hot before filtration, on account of the risk of converting into a less soluble form the barium salts of the acids to be estimated. The barium precipitate is filtered off and well washed with hot water.

The filtrate is evaporated down to 10 cc., and 30 cc. 97 % alcohol are added. The alcoholic solution is adjusted to p_H 7·0 (using phenol red) and 1–2 cc. 10 % $AgNO_3$ solution is added. The silver precipitate contains all the succinic, malic and fumaric acids, but very little lactic acid. The precipitate is filtered off in a Gooch crucible, washed with 75 % alcohol, suspended in water with a few drops of 1 % sulphuric acid and treated with hydrogen sulphide. After removal of the silver sulphide by filtration and of the hydrogen sulphide by aeration, the solution is neutralised with sodium hydroxide and evaporated down to 20 cc. 12 cc. 97 % alcohol are now added, the solution is brought to p_H 7·0, and 10 % $AgNO_3$ is again added. The silver salts of the succinic, malic and fumaric acids are now quantitatively precipitated; in order to estimate their amount, the precipitate is ground with warm water containing a few drops of 50 % H_2SO_4, and the cold solution is titrated with 0·01*N* KCNS, using iron alum in nitric acid as outside indicator.

The silver thiocyanate is filtered off, and in the filtrate the malic acid is estimated polarimetrically as the molybdenum compound, according to the method of Auerbach and Kruger [1923]. The filtrate is neutralised and evaporated down to 8·4 cc., and its rotation taken in a 2 dm. tube. After concentration to 4 cc., 4 cc. of 14·2 % ammonium molybdate solution are added, and 0·4 cc. of glacial acetic acid; when the solution has stood in the dark for 2 to 3 hours, the increase in rotation is determined. Using the mercury green line, it was ascertained that 1 mg. of malic acid gave, under these conditions, a rotation of + 0·21°.

The method was tested by adding known amounts of the acids to muscle, suspended in two volumes of 1·4 % Na_2HPO_4, $2H_2O$, and some results, after subtracting the small quantities found in the control, are shown in Table I.

These figures have been arranged in a diagram (Fig. 2), and it will be seen that, with quantities of malic acid between 45 mg. and 8 mg. added to about 50 g. muscle, the amount recoverable falls off gradually from about 80 % to about 55 %; below 8 mg. only about 35 % is recoverable. The points marked *X* were obtained by the double precipitation method, which gives better results in these regions of low concentration. The low results obtained even

with this method for amounts below 8 mg. cannot be due to the effect of lactic acid, but are probably brought about by adsorption of the barium malate on the barium phosphate precipitate.

Table I.

Muscle used	Amount added	Amount found	% recovered
40 g. beef	43 mg. malic acid	34·0 mg.	79
40 „	21·5 „	13·1	61
40 „	10·75 „	5·8	54
45 „	30 „	24·0	80
45 „	50 „	37·5	75
45 „	10 „	3·7	37
50 „	10 „	3·4	34
50 g. pigeon	8·5 „	5·3	62
50 „	5·6 „	1·8	32
50 „	3·8 „	1·2	32
45 g. beef	30 mg. succinic + 30 mg. fumaric	42·0	70
45 „	„ „	42·0	70
50 g. pigeon	21·7 mg. succinic + 3·9 mg. fumaric	21·0	82
50 „	„ „	15·4	60
50 „	„ „	17·0	66

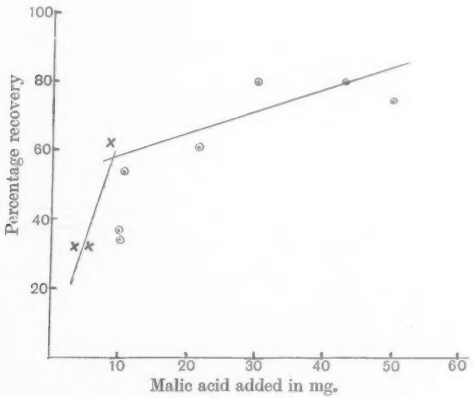

Fig. 2.

In order to test whether the silver precipitate obtained consisted only of succinate, malate and fumarate (one or more of these acids) the silver in weighed amounts was estimated.

(*a*) The silver precipitate obtained in nitrogen weighed 58·7 mg. and contained 65·1 % of silver; the calculated percentage, as the precipitate was found to contain 3·4 mg. of silver malate, is 64·7.

(*b*) The silver precipitate obtained from an initial sample weighed 54·5 mg., and this contained 64·1 % of silver. Allowing for the 5·7 mg. of silver malate contained in it, the calculated percentage was 64·5.

In this method the removal of hydroxy-acids with acid permanganate is avoided; there is therefore no risk, as in the previous method for succinic acid, of the breakdown of possible traces of 5-carbon chain acids to succinic acid in the course of the estimation.

The effect of aerobic and anaerobic conditions on the malic acid content and on the total content of the three acids.

Pigeon breast muscle was used in these experiments, and the arrangement was similar to that in Exps. 3, 4 and 5. As will be seen, the malic acid found per 50 g. muscle always lies below 8 mg., that is, in the region of only about 35 % recovery; in order, therefore, to compare the total amount of acids under the different conditions, this amount of malic acid is doubled, and thus can be compared with the amounts of succinic and fumaric acids found. Of the latter acids, as we have seen, about 70 % is recovered. The fumaric acid is reckoned as present to the extent of 50 % of the malic (according to Einbeck [1919] and Dakin [1922] this is roughly the ratio found at equilibrium when the acids are in the presence of the muscle enzyme). The succinic acid is found by difference.

Exp. 6. Two samples were ground immediately after removal from the animal with a mixture of equal volumes of ice-cold alcohol and ice-cold 0·7 % Na_2HPO_4, $2H_2O$; three other samples remained at 33° in four volumes of 0·7 % Na_2HPO_4, $2H_2O$, one in nitrogen for 3 hours, the second in oxygen for 1 hour and the third in oxygen for 3 hours.

	Malic acid in mg. per 100 g.	
	Found	Corrected
Initial (1)	9·7	19·4
„ (2)	12·0	24·0
In nitrogen	1	2
In oxygen 1 hour	1·5	3
„ 3 hours	1	2

Exp. 7. Temp. 37–39°; each muscle sample in two volumes of 1·4 % Na_2HPO_4, $2H_2O$.

11.45 a.m. Flasks filled with nitrogen.
1.45 p.m. N 1 removed; other flasks filled with oxygen.
2.45 „ O 1 removed; remaining flask filled with nitrogen.
3.45 „ N 2 removed.

In order to inhibit if possible the oxidation of malic acid whilst not interfering with the oxidation of succinic acid to malic acid, the addition of arsenic was tried; according to Batelli and Stern the latter oxidation is not markedly slowed until the concentration of arsenious acid reaches 1 : 1000, while the further oxidation of malic acid is inhibited at a concentration of 1 : 10,000. A fourth flask was therefore exhausted at 12.0 noon and filled with nitrogen; at 2.0 p.m. 15 mg. of arsenious acid (dissolved in a little 0·1 *N* sodium hydroxide) were added, and the atmosphere was changed to oxygen. The flask was repeatedly shaken till 3.0 p.m., when alcohol was added.

An initial sample, precipitated at once with alcohol and an equal volume of phosphate solution, was also worked up.

	Malic acid found in mg. %	Malic acid corrected in mg. %	Total silver in cc. KCNS %	Amounts of acids calculated in Ag ppt. in mg. %	Total acid with malic corrected in mg. %
Initial	6·3	12·6	37	6·3 malic 6·3 fumaric 12·2 succinic	31·1
N 1	1	2	80	1·0 malic 1·0 fumaric 47·8 succinic	50·8
O 1	2·4	4·8	40	2·4 malic 2·4 fumaric 20·6 succinic	27·8
N 2	Nil	Nil	72	44·6 succinic	44·6
With arsenic	2·0	4·0	32	2·0 malic 2·0 fumaric 16·4 succinic	22·4

Exp. 8. This experiment was like the previous one, except that hydrogen, passed slowly through two pyrogallol bottles, was used for obtaining the anaerobiosis. Temp. 35°; three 60 g. lots, each suspended in 130 cc. 1·4 % Na_2HPO_4, $2H_2O$. An initial sample was also worked up.

1.10 p.m. Flasks filled with hydrogen.
3.10 „ H 1 removed; oxygen introduced.
4.0 „ O 1 removed; hydrogen introduced.
4.45 „ H 2 removed.

	Malic acid found in mg. %	Malic acid corrected in mg. %	Total silver in cc. KCNS %	Amounts of acids calculated in Ag ppt. in mg. %	Total acid with malic corrected in mg. %
Initial	9·3	18·6	57·3	9·3 malic 9·3 fumaric 18·0 succinic	46
H 1	1	2	77	1·0 malic 1·0 fumaric 46·0 succinic	49
O 1	2	4	25	2·0 malic 2·0 fumaric 11·8 succinic	17·8
H 2	1·2	2·4	53	1·2 malic 1·2 fumaric 30·7 succinic	34·3

Exp. 9. In this experiment the method of double silver precipitation was used; in other details the procedure was the same as for Exp. 8, except that an extra hydrogen and an extra oxygen period were introduced.

1.0 p.m. Flasks filled with hydrogen.
3.0 „ H 1 removed; oxygen introduced.
3.35 „ O 1 removed; hydrogen introduced.
4.10 „ H 2 removed; oxygen introduced.
4.45 „ O 2 removed; hydrogen introduced.
5.20 „ H 3 removed.

	Malic acid found in mg. %	Malic acid corrected in mg. %	Total silver in cc. KCNS %	Amounts of acids calculated in Ag ppt. in mg. %	Total acid with malic corrected in mg. %
Initial	4	8	41·4	4·0 malic 4·0 fumaric 19·4 succinic	31·4
H 1	1	2	61	1·0 malic 1·0 fumaric 37·8 succinic	40·8
O 1	—	—	6	3·9 succinic	3·9
H 2	1	2	38	1·0 malic 1·0 fumaric 22·9 succinic	25·9
O 2	—	—	4	2·6 as succinic	2·6
H 3	—	—	12·4	8·1 as succinic	8·1

Exp. 10. The power of the minced muscle was tested as regards the oxidation of added succinic and malic acids in the later stages of incubation.

Three samples were kept in hydrogen for 2 hours; then one was removed and precipitated. Oxygen was introduced into the other two, and to one a solution containing 25·5 mg. of succinic acid and 25 mg. of malic acid (neutralised with sodium hydroxide) was added; both flasks were kept in the bath for an hour. Temp. 35°; two volumes of 1·4 % Na_2HPO_4, $2H_2O$ used.

	Malic acid found in mg. per 50 g.	Malic acid corrected	Total silver in cc. KCNS per 50 g.	Amounts of acids calculated in Ag ppt. mg. per 50 g.	Total acid with malic corrected in mg. per 50 g.
H 1	1·3	2·6	32·1	1·3 malic 1·3 fumaric 18·5 succinic	22·4
O 1	0·2	0·4	3·0	0·2 malic 0·2 fumaric 1·65 succinic	2·25
O + 25 mg. malic + 25·5 mg. succinic	5·8	11·6	20	5·8 malic 5·8 fumaric 2·1 succinic	19·5

Discussion.

Perhaps the most important result of this work has been the evidence gained of the removal in oxygen of part of the succinic acid (probably by transformation into carbon dioxide and water, or at any rate into a substance not precipitated by silver in 36 % alcohol) and the renewed formation of the acid in the absence of oxygen. The explanation which may be put forward for the present is that accumulation of the acid up to a certain point hinders further formation; possibly there is a back reaction according to the mass action law. In oxygen when the accumulation is removed renewed formation takes place, and *in vivo* we may suppose that a continuous production and removal go on. As to the nature of the precursor in the muscle, there is at present no information.

Two suggestions may be made as to the cause of the greater removal in oxygen of the three acids at later stages in incubation. One is that oxidation is in some way facilitated; the other that in the early stages production of the acids is more vigorous, falling off later. The latter explanation seems the more

likely. There is no reason to suppose that production ceases in oxygen, and that its activity falls off as time goes on is indicated by the progressively lower maxima on return to nitrogen. Further, according to Batelli and Stern [1911, 2], the power of the muscle to oxidise added malic and fumaric acids, far from becoming greater in course of time, falls off rapidly after 1 or 2 hours. In one experiment in the present work, out of 40 mg. added to 50 g. of muscle at the beginning of the third hour, only 21 mg. had been oxidised away by the end of the hour; the muscle therefore is very near the limits of its oxidising capacity, and the fact that the maximum in nitrogen can be practically completely removed in oxygen at this stage must not be taken as a sign that the muscle can do much more than this. It would be interesting to test the degree of oxidation of quantities of acid added at different times throughout incubation.

When this work was begun, nothing was known of the concentration of malic and fumaric acids occurring in muscle; the amounts of succinic acid under varying conditions had been ascertained [Moyle, 1924], and it was known from the work of Einbeck and of Dakin that, in the presence of the muscle enzyme, the equilibrium between fumaric and malic acids lay at about 70 % of the latter. But the equilibrium concentration under various conditions of succinic acid and its two oxidation products had never been investigated. It was hoped that possibly conditions might be found favourable to the oxidation of succinic acid to malic and fumaric acids, but unfavourable to the oxidation of the latter acids; in this way, by accumulation of these oxidation products, evidence might be obtained of succinic acid metabolism. In spite of the probability, however, that the oxidation of malic and fumaric acids falls off considerably an hour or two after the death of the animal, while the oxidation of added succinic acid can certainly go on vigorously for many hours, it has not so far been possible to obtain any accumulation of malic acid under aerobic conditions. Even the use of arsenious acid had no effect. This is probably because production of the acids falls off.

The disappearance, when the muscle is transferred to nitrogen, of the malic acid initially present shows that the change from succinic acid to malic is a reversible one. This was to be expected from the experiments of Thunberg [1925], of Batelli and Stern [1921] and of Ahlgren [1925] with added malic acid in the presence of methylene blue or of thionine.

The comparatively high concentration of malic acid in the fresh muscle is interesting; probably the more *post mortem* change were avoided, the higher would be the concentration found, as during the necessary manipulation the muscle suffers oxygen deprivation. In this connection, it is interesting to notice that Einbeck [1914] prepared fumaric acid from fresh beef; he obtained a yield of 2·8 mg. per 100 g. of muscle, but considerably more was probably present, as the method he used of silver precipitation in acid solution is not quantitative. The concentration of malic acid in the oxygenated samples never reaches that found in the initial sample; probably the conditions in the living muscle for production and oxidation of the acids cannot be, or have not yet been, successfully imitated *in vitro*.

SUMMARY.

1. A method has been worked out for the estimation of malic acid in muscle, and of the total amount of succinic, fumaric and malic acids taken together.

2. When minced muscle, suspended in buffer solution, is placed alternately under anaerobic and aerobic conditions, the succinic acid content rises in the nitrogen, falls in the oxygen, rises again in the nitrogen, and so on.

3. With similarly repeated changes of atmosphere, the total amount of succinic, fumaric and malic acids taken together rises in anaerobiosis and falls on oxygenation.

4. The malic acid content is highest in the fresh muscle (about 12–18 mg. per 100 g.); it falls to practically nothing in nitrogen, and on admission of oxygen it may rise, but very slightly.

5. When succinic acid disappears in oxygen it is oxidised further than to fumaric and malic acids; and when the succinic acid maximum is renewed in nitrogen, the latter acid is formed from some other source than reversibly from fumaric and malic acids.

6. The results also suggest that production of succinic acid is hindered by accumulation of the acid, and make possible the hypothesis that *in vivo* production and oxidation go on continuously.

I wish to express my sincere thanks to Sir F. G. Hopkins for his most kind interest and encouragement in this work.

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C. QUANTITATIVE STUDIES OF THE NITROPRUSSIDE REACTION IN NORMAL TISSUES AND TUMOURS.

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The analyses described below provide material for a comparison of the glutathione content of normal tissues and of tumours. The investigation consists of two parts concerned respectively with (*a*) human tissues, normal, malignant, and non-malignant; and (*b*) the tissues of the fowl, and the Rous fowl sarcoma. For the sake of simplicity the extractable material giving the nitroprusside test is spoken of here as glutathione, though the possible presence of another SH compound is not excluded.

Human tissues.

Technique. All the organs and tumours named in Table I, with the exception of the placenta, were obtained from the operating theatres of the Cancer Hospital; the material was brought as quickly as possible to the laboratory, dissected and weighed while still warm, and treated immediately by Tunnicliffe's method [1925]. Every effort was made to avoid necrotic material. The tissue was minced with scissors, ground with sand and trichloroacetic acid; the mass was then filtered on a Büchner funnel, and the grinding and filtration were repeated twice. The three filtrates were united, centrifuged and titrated. To ascertain that the extraction of the soluble glutathione was practically complete, the solid material was then ground and filtered a fourth and a fifth time, and the small amount of glutathione present in these filtrates titrated. Finally, a solution of nitroprusside, ammonia and ammonium sulphate was poured over the residue on the funnel to demonstrate the presence of fixed SH groups. For the titration, 2 cc. saturated ammonium sulphate solution, 2 drops strong ammonia, and 5 drops of 1 % sodium nitroprusside solution, not more than 1 hour old, were placed in a series of test-tubes. $N/100$ iodine was run in until 5–10 drops of the titrated fluid transferred to one of these test-tubes produced no colour. The total amount thus removed was shown to be not more than 2 or 3 % of the whole titre. After the end-point for nitroprusside was reached, a few drops of 1 % soluble starch were added, and the titration continued.

In practically every case, a portion of each tissue analysed was reserved for the preparation of histological sections. This is of great importance, for obviously it is essential to attempt an estimate as accurate as possible of the amounts of non-cellular material, fibrotic or necrotic, which are present. Even when every care is taken, it is impossible to be sure that the small pieces taken for section are really fair samples of 10 or 15 g. of tissue. This is the weak point in all such investigations. But if no microscopic examination were made one might for example estimate the glutathione in a scirrhous and in a medullary cancer of the breast, and be unaware that the "cancer" was very largely non-cellular, consisting in the one case chiefly of fibrous, and in the other of necrotic, material containing very few living cancer cells.

The results obtained with human tissues are shown in Table I.

Notes on the microscopic appearances of the tissues analysed.

(1) *Rectum.* One of the most suitable specimens obtainable for a chemical comparison of normal and malignant tissues is the rectum when removed by abdomino-perineal excision for cancer. A considerable length of normal mucous membrane is available immediately adjacent to the malignant tumour which has developed from it. Five such specimens have been examined. When the innermost layers of any part of the stomach or intestine are dissected off in the ordinary way, microscopic examination shows that the material consists of (*a*) mucous membrane, (*b*) muscularis mucosae, (*c*) a variable amount of submucous layer, which is the plane along which the separation takes place. Of these various tissues, the epithelium of the mucous membrane alone is comparable to the cancer, which has developed from it. Examination of a number of sections suggests that, on an average, something like one-half of the material analysed consists of this epithelium. In the cancer, on the other hand, however carefully this may be dissected out, considerable areas of muscle, fibrous and necrotic tissue, comparatively very poor in cells, will be found in sections; in some of these sections the cancer may occupy one-tenth or less of the area. Hence, when the mucous membrane and the cancer thus prepared yield approximately the same amounts of glutathione it seems very probable that the cellular part of the cancer contains more of this compound than does the corresponding cellular part of the normal mucous membrane.

(2) *Breast.* In Case 6 the normal part of the breast consisted very largely of fibrous and adipose tissue, with very little mammary gland; the cancer, on the other hand, was very cellular, and contained a hundred times more glutathione per cent. than did the rest of the breast. This case illustrates very well the great difficulty of obtaining exactly comparable normal and malignant tissues. Cases 7 and 8 showed typical non-maligant tumours; the former, an intracystic papillary fibro-adenoma, was considerably more cellular than the latter, which was a fibro-adenoma of the ordinary type. The more cellular tumour gave a much higher figure for glutathione (85 mg.) than the latter

Table I. *Human tissues.*

Table I. *Human tissues.*

mg. glutathione per 100 g. fresh tissue

	Normal		Tumours			
			Innocent		Malignant	
Rectum						
Case 1	Mucous membrane above tumour	48	—		Columnar-celled cancer	59, 56
	„ „ below „	35				
„ 2	„ „ above „	56	—		Columnar-celled cancer	52
	„ „ below „	47				
„ 3	„ „ above „	50	—		Columnar-celled cancer	51, 55
	„ „ below „	55				
„ 4	„ „ above „	41	—		Columnar-celled cancer	51
	„ „ below „	50				
„ 5	„ „ above „	52	—		Columnar-celled cancer	51
	„ „ below „	45				
Breast						
Case 6	Breast tissue	0·9	—		Spheroidal-celled cancer	91
„ 7	—		Papilloma	85	—	
„ 8	—		Fibro-adenoma	32	—	
„ 9	—		—		Spheroidal-celled cancer, primary	60
					Spheroidal-celled cancer, metastases, axillary glands	154
„ 10	—		—		Spheroidal-celled cancer, primary	32
„ 11	—		—		Spheroidal-celled cancer, „	29
					Spheroidal-celled cancer, metastases, axillary glands	68
„ 12	—		—		Spheroidal-celled cancer, primary	25
					Spheroidal-celled cancer, metastases, axillary glands	38
„ 13	Axillary glands	67	—		—	
	Axillary glands, tuberculous	49				
	Breast, tuberculous	31				
Tongue						
Case 14	Mucous membrane	42	—		Squamous-celled cancer	77, 81
„ 15	—		—		Squamous-celled cancer	70
Uterus						
Case 16	Muscular wall	51, 61	Fibromyoma	50, 63	—	
„ 17	„ „	51	—		—	
Placenta						
Case 18	Placental tissue	37, 42	—		—	
Ovary						
Case 19	—		Papillomata, ovarian cyst	40, 51	—	
Prostate						
Case 20	—		Adenoma	15, 40	—	
Thyroid						
Case 21	—		Adenoma	28, 40	—	
Stomach						
Case 22	Mucous membrane	47, 50	—		—	
Large intestine						
Case 23	Mucous membrane	29, 32	—		—	
Voluntary muscle						
Case 24	Muscle	38	—		—	
„ 25	„	34, 45	—		—	

Pairs of figures indicate duplicate analyses.

(32 mg.). The remaining tumours of the breast were all spheroidal-celled cancers; satisfactory material for comparison, which could be regarded as normal mammary glandular tissue, was not obtained from any of these specimens. In Case 9 the tumour was very cellular in parts; the lymphoid tissue of the axillary glands was almost wholly replaced by carcinoma, with some fibrous interspaces, and these glands gave the highest figure (154 mg.) of the whole series. Case 10 showed cellular islands, with much fibrous tissue; the glutathione content was low (32 mg.). The primary tumour in Case 11 was a scirrhus, with much fibrous tissue, and the glands showed in parts considerable fibrosis; their glutathione content (68 mg.) was lower than in Case 9. In Case 12 the primary tumour showed a considerable amount of fibrous tissue; the invaded glands showed large areas of carcinoma cells, and the low result (38 mg.) is difficult to explain. Case 13 is included for the sake of comparison; it was a tuberculous breast, with involvement of some of the axillary glands. This case was thought to be one of cancer until the sections were examined.

(3) *Tongue.* Both tumours (Cases 14 and 15) were typical epitheliomas, showing very little necrosis. The material taken for comparison, named "mucous membrane" in the table, was the epithelium dissected off with a varying amount of the underlying fibrous tissue.

(4) *Uterus.* The normal muscular wall from which the mucous membrane was removed (Cases 16 and 17), and the non-malignant tumour (fibromyoma, Case 16) developed from it, are very closely similar in structure and show the same glutathione content.

(5) *Ovary.* The tumour was a papilliferous ovarian cyst of the usual type from which the mucoid contents were removed as far as possible; the non-cellular material consists of the fibrous trabeculae upon which the epithelium rests.

(6) *Placenta.* This was a full-time specimen[1]; a considerable part of the material consists of the connective tissue of the villi. The glutathione content is rather low (37 to 42 mg.).

(7) *Prostate.* This adenoma showed fibrous and glandular portions, and possibly the unequal distribution of these accounts for the large difference between the two analyses (15 and 40 mg.).

(8) *Thyroid.* Here also the analyses differ more than is usual. The gland showed distended vesicles, and no papilliferous growth; the very large portion of the sections occupied by colloid suggests that, if the colloid contains no glutathione, the amount of this compound in the epithelium of the vesicles must be very large.

(9) *Stomach* and (10) *Large intestine.* These samples of mucous membrane were dissected off through the submucous coat, as in the specimens from the rectum, and showed similar proportions of cellular and non-cellular material. The former specimen was taken from the healthy portion of stomach wall

[1] We are indebted for this to Dr Holland, of St Luke's Hospital, Chelsea. The analysis was begun 45 minutes after the expulsion of the placenta.

resected in a case of gastric ulcer, the latter from a piece of intestine removed on account of prolapse from a colostomy opening.

(11) *Voluntary muscle.* This was the pectoral muscle removed in amputation of the breast.

The general impression that one obtains from examining sections of almost all these tissues, and from considering at the same time the quantitative chemical results, is that on the whole the amount of glutathione varies with the amount of cellular, or perhaps rather nuclear, material present, and is quite independent of the normal or neoplastic nature of the tissue.

Discussion.

The data in Table I certainly give no indication of any deficiency of glutathione in tumours. Thus in the series of comparative analyses of the mucous membrane, and cancers, of the rectum, the amounts in the former range from 35 to 56 mg., and in the latter from 52 to 59 mg. % in spite of the difficulty, alluded to above, of obtaining this cancer tissue without a large quantity of non-cellular material. The value of the figures for cancers of the breast is lessened by the impossibility of obtaining normal glandular tissue for comparison; but the high figures (154 and 68 mg.) given by axillary glands in which the lymphoid tissue is replaced wholly or largely by cancer cells shows that these must contain a considerable amount of glutathione. Again, the two epitheliomas of the tongue (Cases 14 and 15), which offered particularly compact masses of carcinoma, gave high figures (70 to 81 mg.). Of the innocent tumours, the uterine fibroid (Case 16) gave the same result as the muscular wall of the uterus, with which it is almost identical in structure. But the conclusion to be drawn from this investigation is illustrated most clearly by the fact that no less than 14 out of the 20 largest amounts of glutathione were found in cancers.

Fixed —SH groups.

The solid residue after extraction of the soluble glutathione was in every case tested for insoluble —SH groupings. The intensity of the colour produced bore no constant relation, either direct or inverse, to the amount of glutathione extracted. The residue of the specimen of breast-tissue giving an extractable glutathione content of 0·9 mg. % gave a negative result with this test. When the soluble glutathione values are arranged in order of magnitude the residues of the tissues to which the seven highest figures correspond gave strong reactions for fixed —SH without exception. However, two of the tissues having a glutathione value of 51 mg. % gave a weak and a very strong reaction respectively. Nor if the tissues are grouped according to a histological classification is the agreement between type and residual nitroprusside reaction at all close.

Starch as indicator.

Glutathione values as determined by the starch end-point method gave, in the case of human tissues, figures of the order of 40 to 50 % greater than those obtained with the nitroprusside technique (Table II). The prostatic adenoma, however, yielded starch results which were in the one case twice as great and in another nearly four times greater than the nitro-prusside figures. The nature of the substances which combine with iodine after the nitroprusside end-point is reached has not been determined.

Table II. *Human tissues.*

Comparison of glutathione content (mg. per 100 g. fresh tissue) as determined by nitroprusside and by starch.

N=nitroprusside as indicator. S=starch as indicator.

Case	N	S	Case	N	S	Case	N	S
1	48	60	7	85	130	16	63	75
	35	38	8	32	59	17	51	71
	59	73	9	60	102	18	42	63
	56	72		154	231		37	50
2	56	72	10	32	60	19	51	82
	47	67	11	29	49		40	77
	52	77		68	104	20	40	80
3	50	77	12	25	34		15	55
	55	82		38	55	21	28	43
	51	74	13	31	58		40	52
	55	70		67	109	22	47	68
4	50	69		49	78		50	63
	51	66	14	77	84	23	29	41
	41	55		81	89		32	42
5	52	80		42	52	24	38	48
	45	71	15	70	90	25	45	53
	51	71	16	51	63		34	39
6	91	113		61	69			
	0·9	2·7		50	62			

Concentration of glutathione in human tissues.

The figures in Table I show that the glutathione content of the human tissues examined is rather low (generally from 25 to 60 mg. %) in comparison with the organs analysed by previous workers [Tunnicliffe, 1925; Voegtlin and Thompson, 1926; Holmes, 1926; and Yaoi and Nakahara, 1926], which are chiefly the liver, spleen and kidney of the rabbit, rat and fowl. These latter organs contain generally from 150 to 300 mg %. Certainly one might expect that the organs of animals smaller than man, in which the metabolism per unit body-weight is greater, would show a higher concentration of glutathione. These considerations show the importance of using the corresponding *human* normal tissues as controls on analyses of human tumours.

TISSUES OF THE FOWL, AND THE ROUS SARCOMA.

Forty estimations in all were carried out, and Rous tumours from 27 fowls were analysed. The liver, pancreas, kidney, lung, and voluntary muscle were taken as control tissues. The results are shown in Table III.

Table III. *Tissues of the fowl, and the Rous sarcoma.*

N = nitroprusside as indicator. S = starch as indicator.
mg. glutathione in 100 g. fresh tissue.

Fowl	Inoculation site	Rous tumour N	Rous tumour S	Liver N	Liver S	Pancreas N	Pancreas S	Muscle N	Muscle S	Kidney N	Kidney S	Lung N	Lung S
1	Intramuscular	0·0	20	85	106	32	80	13	16	65	83	—	—
,,	,,	3·3*	9·6*	—	—	—	—	—	—	—	—	—	—
2	,,	12	14	151	194	21	48	16	18	98	114	—	—
,,	,,	16*	24*	—	—	—	—	—	—	—	—	—	—
3	,,	34	54	136	154	36	59	24	26	97	97	—	—
4	,,	24	27	175	195	61	95	15	21	—	—	—	—
5	Subcutaneous	3·1	—	128	143	53	81	17	19	130	158	—	—
6	,,	0·0	21	—	—	—	—	—	—	—	—	—	—
7, 8, 9	,,	13	18	—	—	53	86	—	—	—	—	39	51
10	,,	34	47										
11	,,	19	29										
12, 13	,,	17	24	—	—	46 (6 fowls)	87	—	—	—	—	—	—
14	,,	17	30										
15	,,	14	21										
16, 17, 18	,,	28	41	—	—	—	—	—	—	—	—	—	—
19, 20, 21, 25, 26	,,	28	52			40 (12 fowls)	76	—	—	—	—	—	—
22, 23, 24, 27	,,	36	51										

* These tumours to the naked eye appeared especially necrotic.

Technique. In 17 estimations on the Rous tumours, sometimes pooled, of 27 fowls, the amounts of glutathione found ranged from nil to 36 mg. %. The first six samples of tumour analysed were produced by inoculation in the manner which has for some reason become conventional in dealing with the Rous tumour, namely, by injection deeply into the pectoral muscle. The material obtained thus is a mixture of living and necrotic tumour, mucoid ground-substance, and disintegrated muscle, and from this it is impossible to isolate the healthy tumour tissue required for analysis. The amounts of glutathione are very irregular (nil to 34 mg.), and it is impossible to take from such material pieces for microscopical examination which are fair samples of the whole. It was found very much better to inoculate the tumour in the way almost always employed for rat and mouse tumours, that is, subcutaneously. The tumour after inoculation under the skin of the breast spreads as a sheet of fairly compact cellular tissue over the pectoral muscle, which it does not at first invade. The sheet soon invades the skin, but is easily scraped off it, and in sections is found to contain no muscle, very little necrotic material, and a comparatively small proportion of ground substance. The tumour is in a suitable state for examination 6 days after inoculation in fowls weighing 300–400 g., and in 11 days in older birds (1000–1500 g.). The amounts of glutathione found in the tumours grown subcutaneously range from 3 to 36 mg. %; the single case (No. 6) in which none was found was a bird which died during the night and was examined on the following day. The tumour grows freely in White Leghorns, which were used in most of these experiments. The birds were bled under anaesthesia before the removal of the various tissues.

Discussion.

Of the five control tissues, the liver gives by far the highest results (up to 175 mg.). The wide range of variations in these tissues (liver, 85 to 175 mg.; pancreas, 21 to 61 mg.; kidney, 65 to 130 mg.) is similar to that found in the organs of rats by Voegtlin and Thompson (*e.g.* kidney, 19 to 156 mg.; liver, 135 to 261 mg.). Table III shows clearly that there is nothing whatever peculiar about the glutathione content of the Rous tumour. Of the 17 samples of it analysed, 11 gave a figure higher than the lowest amount found in voluntary muscle (13 mg.), and no less than 6 contained more than the least amount in the pancreas (21 mg.), which latter is certainly a very cellular organ. Yaoi and Nakahara [1926] have maintained recently that the Rous tumour "is very characteristic in showing no, or exceedingly feeble response to the nitroprusside test. The tumour is in this way readily separable from other malignant tumours as well as from normal tissue," and they have suggested that this absence or great deficiency of glutathione is associated with the resistance of the tumour to drying and to the action of glycerol. The results given in Table III suggest that the Japanese workers have been misled by using the glutinous and largely non-cellular material which can be obtained in abundance when a Rous tumour inoculated into muscle reaches an advanced stage of growth. They make no mention of any microscopical control of their chemical work. From Tables I and III one sees that a Rous tumour may contain as much glutathione as is present in the pancreas of the fowl bearing it (fowl 3) or in some normal human tissues (rectal mucous membrane, voluntary muscle, placenta).

Incidentally, the results show that glutathione estimations have no bearing on the question whether the Rous tumour is or is not similar in nature to mammalian tumours.

Fixed —SH groups. After extraction of the Rous tumour the residue was tested for —SH groups. With two exceptions (the tumours from fowl 4 and the pooled tumours from fowls 16, 17 and 18) a distinct to strong colour was observed. In the two exceptions only a weak colour resulted. The extracts from these two tumour specimens gave a moderately large yield of glutathione, namely, 24 and 28 mg. %. Here again as in the case of the human tissues there was found no constant correspondence between extracted glutathione and residual —SH groupings. The tumour with a glutathione value of only 3·1 left a residue which gave practically no colour with the reagents. In many of these tests the colour obtained was of an extraordinarily fugitive nature.

Starch as indicator. In most of the analyses the difference between the results given by nitroprusside and by starch is similar to that found in the case of human tissues. But two instances are peculiarly instructive. In fowls 1 and 6, titration with nitroprusside gave nil, and with starch 20 or 21 mg. % "glutathione" (Table III). Yaoi and Nakahara, who used starch alone as indicator in all their quantitative estimations, found 20 mg. % as a maximum

in the Rous tumour. But as their qualitative nitroprusside tests were almost or quite negative, it is clear that their quantitative results with starch are quite unreliable as estimations of glutathione.

Glutathione and anaerobiosis.

It has been stated that a deficiency or absence of glutathione in tumours is in accordance with Warburg's observations on anaerobiosis in tumours, and corroborates his teaching. But, apart from the fact that such deficiency of glutathione does not occur, one may point out that it is not required at all by Warburg's theory of malignancy. He has never claimed that tumour cells do not oxidise fats or amino-acids; and in a recent paper [Warburg, Wind and Negelein, 1927] has calculated that not more than two-thirds of the carbohydrate used by a tumour is dealt with anaerobically. Hence in a tumour the metabolism of fats, of proteins, and of one-third of the carbohydrates, might be carried out by those same factors, including glutathione, which are active in normal tissues.

SUMMARY.

Analyses of normal tissues and of tumours in man, and in the fowl, show that tumours are not distinguished by any deficiency in glutathione.

We are much indebted to Dr H. J. B. Fry, Pathologist to the Cancer Hospital, in whose laboratory the sections of human tumours were prepared and examined.

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CI. OBSERVATIONS ON THE RENAL THRESHOLD FOR GLUCOSE.

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In the course of a recent investigation on the blood-glucose changes in general anaesthesia, certain observations were made which have a direct bearing on the question of the so-called renal threshold for glucose.

The renal threshold may be defined as being that level or concentration of sugar in the blood which has to be reached or exceeded before any easily recognisable quantity of sugar will appear in the urine. The idea of a renal threshold for a substance presupposes that until a certain concentration shall have been reached in the blood none will appear in the urine.

The prevailing opinions on this subject may be briefly summarised as follows: Macleod [1926] considers the renal threshold for sugar to lie between 0·160 % and 0·180 %; Todd [1923] from 0·170 % to 0·180 %; MacLean [1925] from 0·160 % to 0·170 %; de Wesselow [1925] regards the normal as 0·180 %, but he recognises considerable variations above and below this figure. Host [1925], in an interesting study on carbohydrate tolerance in pregnancy, has shown that, while early in pregnancy the renal threshold is normal, later on the threshold falls and permits glycosuria to occur independently of hyperglycaemia. Goto and Kuno [1921] placed the upper level of the renal threshold at 0·170 %, but they were unable to state the lower level. Benedict, Osterberg and Neuwirth [1918] deny the significance of a renal threshold, stating that their results (on two normal men) indicate that the threshold doctrine should be abandoned. Most of the foregoing workers have based their conclusions generally on studies of carbohydrate tolerance, or on the giving of standard doses of glucose; in other words, their studies are based on the behaviour of exogenous glucose.

Certain difficulties stand in the way of a correct estimation of the renal threshold: (*a*) even when blood and urine are removed as nearly at the same time as possible they cannot be considered as simultaneous specimens, (*b*) while a blood-sugar curve is falling the relationship between the sugar concentration in the blood and in the urine may be quite different from what it is while the curve is ascending, (*c*) the rate of urine formation is an unknown and variable but important factor, (*d*) it is difficult (unless blood is removed very often, *e.g.* at 2-minute intervals) to make quite certain about the highest point of a curve, *e.g.* after a glucose meal, or during or after anaesthesia.

Method of investigation.

It was decided to approach this subject from a different view-point, and to study the behaviour of endogenous, as opposed to exogenous, sugar in the blood. For some time it has been generally known that a marked hyperglycaemia may be caused by the administration of a general anaesthetic, such as chloroform or ether, the hyperglycaemia being presumably a manifestation of the glycogenolytic action of the anaesthetic which causes a "mobilisation" in the sugar depôts of the body. The source of the hyperglycaemia is thus endogenous, unlike that caused by a glucose meal.

The method of investigation chosen was as follows. Forty-four successive subjects, for operation under general anaesthesia by ether or chloroform-ether mixture, had their blood-sugar estimated at intervals of 15–20 minutes, before, during and after anaesthesia. The cases were not selected in any way, and none of them was known to suffer from diabetes or from renal trouble. The variety of the lesions for which operation was performed was characteristic of that found on the surgical side of any large general hospital. In the case of each patient a blood-sugar curve was drawn up, and the curve in Fig. 1

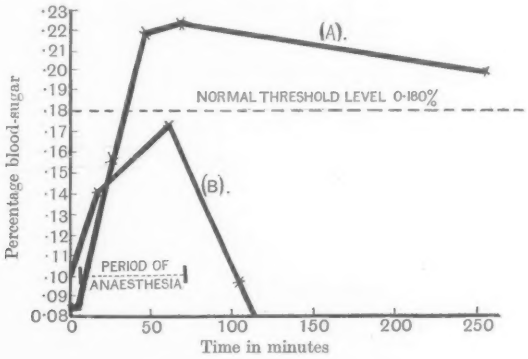

Fig. 1.

(A) Blood-sugar curve of case No. 3.
A. W., *aet.* 35 years. Hydatids in liver.
Open ether anaesthesia for 70 minutes.
Sugar absent from urine both before and after operation.

This curve, which is quite typical of the blood-sugar alterations in anaesthesia, may be compared on the same scale with

(B) Blood-sugar curve after 50 g. glucose meal as carried out by MacLean [1925].

(case 3) is characteristic of the curves in the 44 cases under consideration. On this graph there is also shown, for comparison, the curve of the blood-sugar after a 50 g. glucose test meal in normal individuals as carried out by MacLean [1925]. All the essential details, such as nature of the anaesthetic chosen (mainly ether), period of administration, nature of disease and of operation,

age of patient, etc., were noted and tabulated. In addition, the first urine passed after recovery from the anaesthetic was collected and examined for sugar by (1) the fermentation test, and (2) Bertrand's modification of Fehling's test. The pre-operative urine was also examined for sugar and in each case was found to be negative. The initial and maximum blood-sugar percentages were noted, and, in addition, an approximate estimate was made of the length of time during which the blood-sugar might reasonably be considered to be at a hyperglycaemic level. This level was fixed arbitrarily at 0·180 %.

The essential results are shown in Table I.

Table I. *To show increase in blood-sugar due to anaesthesia, the period in minutes during which the blood-sugar was above* 0·180 %, *and the presence or absence of post-operative glycosuria.*

Number in series	% B.S. before operation	Maximum % B.S.	Minutes above 0·180 %	Sugar in urine	Number in series	% B.S. before operation	Maximum % B.S.	Minutes above 0·180 %	Sugar in urine
1	0·177	0·297	100	+	23	0·109	0·187	5	+
2	0·122	0·241	10	+	24	0·143	0·253	200	+
3	0·083	0·222	220	−	25	0·160	0·181	.	+
4	0·115	0·196	.	+	26	0·095	0·187	15	+
5	0·146	0·386	250	−	27	0·090	0·184	.	−
6	0·160	0·216	45	+	28	0·100	0·211	35	−
7	0·146	0·181	.	+	29	0·106	0·156	.	+
8	0·137	0·206	60	−	30	0·100	0·287	50	+
9	0·184	0·227	30+	+	31	0·050	0·143	.	+
10	0·100	0·216	5	+	32	0·118	0·156	.	+
11	0·150	0·184	5	+	33	0·075	0·181	.	−
12	0·120	0·181	.	−	34	0·118	0·175	.	+
13	0·093	0·243	140	−	35	0·098	0·133	.	+
14	0·118	0·156	.	+	36	0·165	0·206	25	−
15	0·175	0·320	200	+	37	0·110	0·175	.	+
16	0·141	0·156	.	+	38	0·143	0·193	15	−
17	0·162	0·266	50	−	39	0·118	0·137	.	+
18	0·088	0·214	10	−	40	0·112	0·150	.	+
19	0·099	0·137	.	+	41	0·106	0·237	60	+
20	0·109	0·225	200	+	42	0·037	0·193	5	+
21	0·109	0·227	45	+	43	0·110	0·175	.	−
22	0·106	0·147	.	−	44	0·191	0·291	180	+

Discussion.

It is necessary now to examine this table in detail. Of the 44 cases 30 showed glycosuria and 14 did not.

(A) Of the 30 glycosuric cases, in 11 the blood-sugar never reached 0·180 %, *i.e.* in 37 % of cases sugar was excreted by the kidney at a level below the normal threshold value. Of the 14 cases where no sugar was excreted, in 12 cases (*i.e.* in 86 %) the blood-sugar had risen above the normal threshold of 0·180 %. Thus 37 % in the first case and 86 % in the second do not seem to accord with the doctrine of the renal threshold. It may be objected, however, that in a number of cases the blood-sugar may have been above 0·180 % for such a short period that practically no sugar would be excreted in that time. To overcome this legitimate objection a further analysis has been made.

(B) Taking those cases where the blood-sugar as shown on the curves

lay above 0·180 % for over 20 minutes one finds that of 17 such cases in 7 (*i.e.* 41 %) there was no glycosuria—still too high a percentage to permit the wholehearted acceptance of this theory. Further, of the 13 cases where the blood-sugar was continuously under 0·180 %, 11 were found to excrete sugar, *i.e.* 87 %.

Therefore, when an arbitrary average figure of 0·180 % is taken as the renal threshold for sugar, and when the results are considered with relation to that figure, one fails to get any weighty evidence in favour of the theory and finds much to gainsay its validity.

(C) If, instead of taking the renal threshold as 0·180 %—a fixed level—one considers only those cases outside the average range of renal thresholds as given by the workers quoted earlier—say 0·160 % to 0·190 %—then the findings are as follows. Of 30 cases of glycosuria, in 14 the blood-sugar was above 0·190 % and in 7 it was below 0·160 %, *i.e.* 14 are in conformity with the theory and 7 are against. Of the 14 sugar-negative cases 9 had a blood-sugar above 0·190 %, and 4 below 0·160 %. This gives 4 cases in consonance with the theory compared with 9 against it.

In other words, no matter how the foregoing table is analysed, whether the renal threshold is taken as a fixed level of 0·180 %, or as a fixed range of from 0·160 % to 0·190 %, and whether the time factor is included or omitted, no positive substantial evidence can be given to support the theory. Rather may it be said that the weight of the evidence is all against it. Case No. 5, for example, had a blood-sugar curve which rose from 0·146 % to 0·386 %, received an anaesthetic for 85 minutes and then a rectal saline with 5 % glucose, and had a blood-sugar concentration over 0·200 % for over 4 hours, and yet the first urine passed when consciousness was regained neither reduced Fehling's solution nor fermented with yeast.

The deduction made therefore from this analysis is that the results in half of the cases examined do not accord with the doctrine of a renal threshold for sugar of about 0·180 %. When, in addition, it is recollected that traces of sugar or related carbohydrates can be demonstrated in normal urine, a further doubt is thrown upon the validity of the theory, so far as sugar is concerned. The nature of the evidence here adduced, while not consistent with the present idea of a sugar threshold, is not sufficiently strong to enable one to submit another theory as attractive, but it demonstrates the need for some re-statement. For the moment, then, it is suggested that the present position should be summarised thus. The renal threshold for sugar, if there is one, is a much more variable factor than is generally supposed. Under the influence of an anaesthetic, it varies greatly between one normal person and another. It even appears to change during the course of a pregnancy [Host, 1925]. For all the evidence to the contrary, it may even vary frequently to slight degrees in the normal person at different times and in different circumstances. No physiological fluids in the body have an absolutely constant composition, but appear to be influenced, if only slightly, by various physical and metabolic

processes and influences in the attempt to maintain a condition of equilibrium. The renal threshold for sugar is not unlikely to be one such varying factor.

SUMMARY.

An investigation is described carried out with the object of determining the relationship between post-anaesthetic glycosuria and the blood-sugar changes due to the anaesthetic. The explanation of the results obtained in terms of the doctrine of the renal threshold for sugar at about 0·180 % proving unsatisfactory, it is suggested that this theory requires re-statement, if not, indeed, abandonment.

This investigation, which was suggested to me by Prof. E. P. Cathcart, F.R.S., was carried out in the biochemical laboratory of the Wolverhampton and Staffordshire General Hospital by kind permission of Dr S. C. Dyke, Pathologist to the Hospital, for whose friendly advice and criticism I am much indebted. Mr Deanesly, B.Sc., F.R.C.S., and Mr Cholmeley, F.R.C.S., Honorary Surgeons to the Hospital, kindly accorded me every facility in their theatres and wards, while all members of the anaesthetics staff gave me their cordial co-operation.

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